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HEMATOPOIETIC STEM CELL AND ITS GROWTH FACTOR

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Rheusus monkey is a good experimental system for man in bone marrow transplantation. Attempts were made to adopt the human progenitor culture system to the rhesus bone marrow. Conditioned medium from phytoagglutinin stimulated human lymphocytes was suitable for the growth of rhesus progenitors in the standard methylcellulose culture assay. The major modification was the need to read BFU-E and CFU-GEMM at day 12 due to the degeneration of cells in the colonies. Monoclonal antibodies against human nonlymphoid leukemia cell lines which have reactivities against human progenitors did not show significant cross-reactivities against the rhesus marrow. Monoclonal antibodies to rhesus bone marrow cells were made. Although no antibodies specific for the stem cells were obtained, an antibody reactive with lymphoid as well as more mature myeloid cells was useful to enrich for progenitor cells. The bone marrow cells depleted of reactive cells to the monoclonal antibody was functional in vivo in that they reconstituted the bone marrow of irradiated autologous and allogeneic recipients. Monoclonal antibodies against rhesus T cells and B cells were also established. These anti-bone marrow and anti-lymphocyte antibodies may be useful for further studies of the rhesus model system. Keywords: Hybridoma, Hemopoietic cells, Hemopoiesis (AW)

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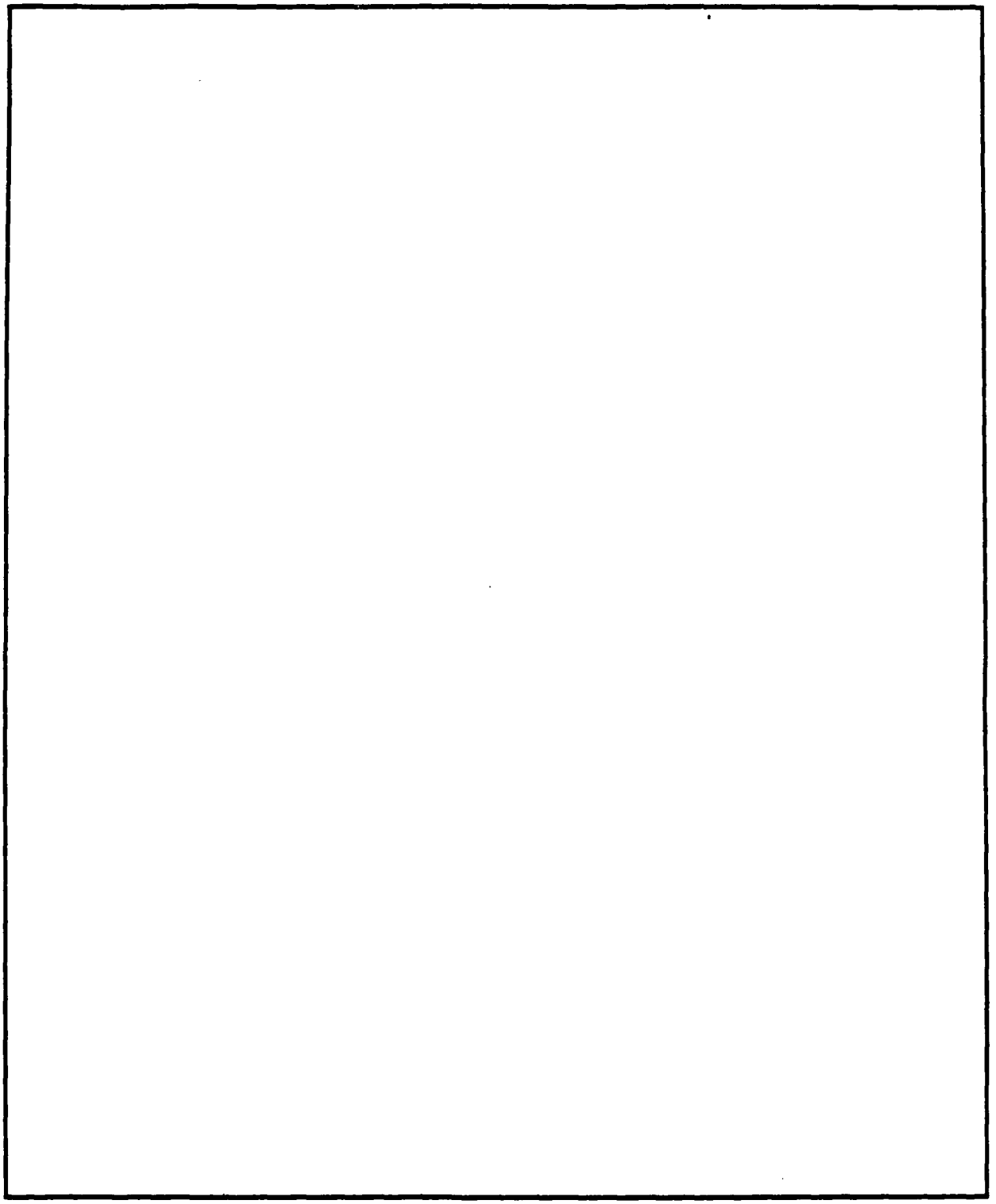

SUMMARY

The work was undertaken to generate monoclonal antibodies against rhesus hematopoietic progenitor cells and to use the human T-T hybridoma system to determine the possibility to detect newer progenitor growth promoting activities. Initially, the standard progenitor cell assay in methylcellulose semisolid medium with the addition of erythropoietin and conditioned medium from human lymphocytes stimulated with phytoagglutinin (PHA) was adopted for the rhesus marrow. The human PHA-LCM was active to support rhesus BFU-E as CFU-GEMM progenitors. Because of cellular degeneration, assays for progenitors of BFU-E and CFU-GEMM were optimally read 11-12 days after the culture initiation. A panel of monoclonal antibodies against human nonlymphoid leukemic cell lines were characterized. Some of them were reactive with progenitors of human CFU-GM and BFU-E. Except for one monoclonal antibody H5, no significant cross-reactivity was detected in rhesus marrow. Monoclonal antibodies to rhesus bone marrow cells were made. Over 1200 hybridoma supernatants were screened for their ability to stain rhesus bone marrow cells. Although several mAb were thought to be of interest, the time consuming and labor intense nature of the approach involving repeated cloning and assays involved the studies of a few antibodies were carried out. One monoclonal antibody, mAb BM25, was shown to be reactive with the majority of peripheral mononuclear cells as well as the majority of the mature bone marrow cells. Depletion of reactive cells from rhesus bone marrow enriched the number of progenitor cells by 6-8 fold. Bone marrow depleted of $BM25^{+}$ cells was able to reconstitute in autologous and allogeneic irradiated recipients. Another monoclonal antibody $SBA^{-}73$ was thought to be reactive with a stem cell fraction because SBA^{-} 73 reactive cells contained the majority of the progenitor cells of BFU-E, CFU-GEMM in initial 4 experiments. With additional 6 monkeys studied, only two of them provide confirmatory results. Thus, the possibility that $SBA^{-}73$ is polymorphic may be accountable for the results. Monoclonal antibodies with pan T cells, cytotoxic/suppressor T cell and B cell specificities were established. T64, an IgM monoclonal antibody against pan T cells, was useful to deplete T cells from bone marrow for allogeneic transplantation. Although the rhesus monkey is a good model for bone marrow transplantation, the limited available bone marrow from a single monkey makes it a difficult model for the generation of monoclonal antibodies against stem cells. The use of the human T-T hybridoma system was encountered with many difficulties. The marked instability of the T-T hybridomas made it a non feasible experimental system.

PREFACE

The experiments reported involved animals and bone marrow donors from normal volunteers. The protocol for marrow donation was approved by the Institutional Review Board of the Oklahoma Medical Research Foundation. Research was conducted according to the principles enunciated in the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources, National Research Council. Permission was obtained for use of copyrighted material.

TABLE OF CONTENTS

Section

LIST OF ILLUSTRATIONS

Figure

LIST OF TABLES

LIST OF TABLES (CONTINUED)

SECTION 1

INTRODUCTION

The work undertaken was to identify hematopoietic stem cells and their growth factors in rhesus monkey. The rhesus monkey model has been considered to be an excellent one for human bone marrow transplantation. The approach was to use monoclonal antibody technology to generate antibodies reactive with rhesus monkey T cells and bone marrow cells. These antibodies may be useful in the identification of stem cells as well as in the treatment of bone marrow to avoid the clinical troublesome graft vs. host disease. Newer B cell growth and differentiation factors have been generated by human and murine systems by T-T hybridoma technology. It was hoped that human T-T hybridomas with stem cell growth promoting activities could be generated.

SECTION 2

OBJECTIVES

2.1 ESTABLISH IN VITRO CFU-GEMM ASSAY FOR RHESUS MONKEY BONE MARROW.

We were able to generate conditioned medium which was able to support the CFU-GM, BFU-E and CFU-CGMM generation by the human bone marrow in methylcellulose with the addition of erythropoietin. The conditioned medium was generated by stimulation of pooled human mononuclear cells from peripheral blood with 1% PHA. It was determined that supernatants from day 1, 3 and 5 cultures were equally active when added in the final concentration of 5%. This conditioned medium will be referred to as PHA-LCM.

2.1.1 METHODS.

The assay method was that described by Ash, et al. (Ash, R.C., Detrick, R.A. and Zanjani, E.D., Blood 58:309, 1981). The bone marrow cells were suspended in Iscove modified Eagles medium with 0.9% methylcellulose, 30% fetal bovine serum of a selected lot from Hyclone (Logan, Utah), and 10% PHA-LCM; $5x10^{-5}$ M 2-mercaptoethanol and 1 U of erythropoietin. Cultures were set up in quadruplicate with cell densities at 10^5 and 10^4 per plate of 35mm plastic dishes. We were able to isolate the colonies by picking them with thinly drawn Pasteur pipettes under microscopic examination. The cells in the colonies were stained and the cell lineages were determined.

2.1.2 RESULTS.

After examining 164 colonies, a good correlation between the morphology of the colonies and the cell types seen in the smear was established. We could reliably identify the mixed cell nature of the CFU-GEMM under 100-200X magnification. This culture condition was used with rhesus bone marrow aspirated from the femur or the iliac crest. The results of three experiments are summarized in Table 1. Several points have emerged from these studies.

Table 1. Formation of CFU-G, CFU-E and CFU-GEMM by bone marrow cells of rhesus monkeys

Monkey Identification Number	Number of Colonies/10^5 Cells Plated		
	CFU-G	CFU-E	CFU-GEMM
32	78 (76,99,65)	125 (100,120,110,170)	72 (55,60,70,73)
33	31 (25,32,36)	54 (46,56,60)	33 (55,35,23,18)
38	0	46 (39,54)	11 (8,14)

In the human system, BFU-E and CFU-GEMM assays are counted 14-21 days after plating the cells. When we counted the monkey bone marrow cultures at day 14, degenerative cells were noted. By day 21, most cells except for histocytes were degenerated and the colonies could be accurately read. We determined that day 11 or 12 would be appropriate days for counting BFU-E and CFU-GEMM in the rhesus system.

We compared the conditioned medium generated by PHA stimulation and mixed lymphocyte reaction of peripheral blood mononuclear cells from monkey and man. In general, the conditioned medium, PHA-LCM from human was more superior in these assays. Because of the convenience and less expense involved in obtaining leukopaks, human PHA-LCM was used in subsequent experiments. We also tried to determine whether different leukopaks provided more potent LCM. In two efforts to generate PHA-LCM, we found both batches had comparable activities. We generated PHA-LCM from several leukopaks, allogeneic mixed leukocyte reaction may play some role in the generation of growth factor promotion activities.

The number of colonies varied from one monkey to another. Repeated cultures from a single monkey indicated similar variation from one aspirate to another. The exact explanation was not apparent. It was likely due to varying amounts of contaminating blood. It is evident that variations between plates of the same bone marrow specimen occured. It was also apparent that only a limited number of aspiration from a young monkey could be done with good yield. This is a major limitation when a large amount of bone marrow is needed for assaying monoclonal antibodies.

2.2 CHARACTERIZATION OF MONOCLONAL ANTIBODIES AGAINST HUMAN NONLYMPHOID LEUKEMIA CELL LINES AND THEIR CROSS-REACTIVITY WITH RHESUS BONE MARROW CELLS.

We generated and characterized eight monoclonal antibodies against human nonlymphoid leukemia cell lines. These antibodies were selected from over 2000 hybridomas by their reactivities against rather restricted cell populations. The antibodies are listed in Table 2 and Table 3 summarized their staining characteristics. Several cell populations of purity greater than 90% were isolated from peripheral blood. ML143, K15, H8, U2 and H5 were expressed on monocytes and granulocytes. H5 and K15 had broader cellular distribution. Four antibodies, H4, H5, H124 and H229 stained platelets. Only H124 was expressed by mature erythrocytes.

H8, U2, H5 were expressed by CFU-GM progenitor cells. K15 and H124 were expressed by both CFU-GM and BFU-E progenitor cells. The remaining four antigens were not expressed by either progenitor cells. It was of some interest that both K15 and H5 were selectively retained by mature eosinophiles but not by other granulocytes. These results were obtained by the isolation of cells either reactive or nonreactive with the bone marrow cells by an immune rosette method or by the depletion of reactive cells by mAb plus complement when the monoclonal antibody was able to fix complement.

2.2.1 METHODS.

Bone marrow cells were obtained from normal volunteers. They were separated by Ficoll-Hypaque density centrifugation to remove erythrocytes and mature granulocytes. In the immune rosette method, 2×10^7 bone marrow cells were incubated with the supernatant containing the mAb under investigation for 30 minutes at 4°C. After three washings with RPMI 1640 and 10% FBS, the bone marrow cells were incubated with 10% human AB red cells coated with $F(ag')_2$ fragments of affinity purified goat antibodies specific for mouse Ig for 30 minutes at 4°C. The rosetting cells were separated from the non-rosetting cells by Ficoll-Hypaque centrifugation. The red cells in the pellet were lysed with buffered $(NH_4)_2SO_4$. For the complement mediated cytolysis procedure, 0.5 ml of 2×10^7/ml of bone marrow cells were incubated with 0.5 ml of hybridoma culture

Table 2. Monoclonal antibodies against human nonlymphoid cell lines

Monoclonal Antibodies	Molecular Weight	Ig Class	Immunogen
ML143	145/180KD	IgM	ML-I (A myelogenous leukemia line)
K15	21KD	IgG3	K562 (A CML in blast crisis line, erythroid)
H4	140-100KD	IgG2b	HEL (An erythroleukemia cell line)
H5	26KD	IgG2b	HEL
H8	65-74KD	IgG2	HEL
H124	95/150KD	IgM	HEL
H229	62KD	IgG2b	HEL
U2	180-190KD	IgG1	U937 (A histocytic lymphoma line)

Table 3. Staining characteristics of monoclonal antibodies against nonlymphoid cell lines

Antibodies	Granulocytes	Monocytes	Lymphocytes	Erythrocytes	Platelets
M143	> 90%	40-60%	neg	neg	neg
K15	neutrophils: neg eosinophils: +	>90%	>95%	neg	neg
H4	weakly +	>90%	neg	neg	++
H5	5-15%	>95%	10-20%	neg	++
H8	>95%	>95%	neg	neg	neg
H124	neg	neg	<10%	++	++
H229	neg	neg	neg	neg	+++
U2	>90%	>95%	neg	neg	neg

supernatant for 30 minutes at 37°C. 1.0 ml of baby rabbit complement (Pel Freez Biologicals, Rogers, AR) was added and the mixture was incubated for 45 minutes at room temperature. Dead cells were removed by centrifugation on a Ficoll-Hypaque gradient. Bone marrow cells were cultured in quadruplicates in Iscove's modified Dulbecco's medium containing 0.9% methyl-cellulose, 10% conditioned medium from human peripheral blood leukocytes stimulated with phytohemagglutinin (PHA-LCM), 30% FBS. One unit of erythropoietin (sheep fraction III Connaught, Toronto, ONT) was added to each plate. BFU-E were scored as hemoglobin-containing single or multiple colonies of greater than 64 cells on the 14th day. This culture also allowed CFU-GM colony formation. CFU-GM were scored as colonies of greater than 40 cells on the 14th day.

2.2.2 RESULTS.

The studies on mAb ML143, H8 and U2 have been published (Takaishi, T. and S.M. Fu, J. Immunol. 135:1523, 1985; see Appendix A, this report). Tables 4 and 5 summarize the data which show that H8 and U2 were expressed on CFU-GM. ML143 was not expressed on CFU-GM. None of these three antibodies were reactive with BFU-E progenitors.

Table 4. Expression of antigens H8 and ML143 on BFU-E and CFU-GM as determined by C-mediated lysis

Experiment	% Dead Cells	Number Colonies/10^5 Cells Plated BFU-E	CFU-GM
1. Untreated	0.9	88.5 ± 14.0[a]	45.8 ± 7.0
C alone	1.1	105.8 ± 3.5	52.0 ± 6.7
mAb H8 + C	22.3	84.0 ± 14.8	4.7 ± 0.5
2. Untreated	0.8	59.0 ± 11.0	70.7 ± 14.6
C alone	0.9	49.0 ± 13.0	60.0 ± 7.1
mAb H8 + C	36.1	74.0 ± 13.1	0
3. Untreated	2.9	88.0 ± 14.0	46.0 ± 7.0
C alone	2.8	106.0 ± 4.0	52.0 ± 7.1
mAb ML143	23.6	80.2 ± 20.3	50.2 ± 15.7
4. Untreated	1.8	242.5 ± 12.2	76.2 ± 6.3
C alone	2.3	224.1 ± 35.4	83.4 ± 5.3
mAb ML143	53.9	311.0 ± 36.7	155 ± 19.5

[a]Data are presented as the mean ± standard deviation of quadruplicate plates.

Table 5. Bone marrow CFU-GM progenitor cells expressed antigens H8 and U2 but not antigen ML143; none of these antigens were expressed by BFU-E progenitors

Bone Marrow Cell Type	BFU-E/10^5Cells Plated I	BFU-E/10^5Cells Plated II	CFU-GM/10^5Cells Plated I	CFU-GM/10^5Cells Plated II
Unseparated	630 ± 65[a]	275 ± 16	100 ± 11	77 ± 15
$H8^+$	53 ± 5	58 ± 11	160 ± 27	111 ± 23
$H8^-$	677 ± 38	225 ± 27	28 ± 5	12 ± 1
HLA-DR^+	539 ± 153	522 ± 132	125 ± 2	120 ± 7
HLA-DR^-	3 ± 2	1 ± 1	1 ± 0	2 ± 1
Unseparated	459 ± 27	380 ± 23	96 ± 10	77 ± 12
$U2^+$	23 ± 16	26 ± 21	206 ± 54	137 ± 14
$U2^-$	441 ± 61	419 ± 58	40 ± 16	32 ± 17
HLA-DR^+	1493 ± 98	1396 ± 246	461 ± 127	191 ± 39
HLA-DR^-	9 ± 8	3 ± 5	5 ± 4	1 ± 1
Unseparated	192 ± 28	213 ± 27	65 ± 15	93 ± 19
$ML143^+$	68 ± 19	55 ± 11	16 ± 13	32 ± 7
$ML143^-$	283 ± 21	239 ± 37	95 ± 13	129 ± 23

[a]Data are presented as the mean ± standard deviation of quadruplicate plates.

Bone Marrow cells which were isolated by the immune rosette method were stained by Wright-Giensa staining and the reactive cells were identified. The results showed that mAb H8 and U2 were reactive with myeloid lineage cells at different stages of development. ML143 was reactive with more mature cells and only a subpopulation of myeloblasts was stained. None of these antibodies were reactive with erythroblasts and megakaryocytes. These results agreed with the data in Tables 4 and 5. ML143 was also reactive with ConA activated T cells while H8 and U2 did not. The diffuse bands brought down by ML143 would suggest that it was reactive with a carbohydrate portion of glycopeptides. Thus, H8 and U2 are myeloid lineage specific.

K15 and H124 are expressed by CFU-GM and BFU-E. K15 is a 21KD antigen. mAb H124 brought down a band at 130KD from platelets and a complex of 140-150KD/90-94KD from HEL cells. Because of the unusual reactivity and the possibility that the active site was a carbohydrate, with the help of Dr. L.R. Lamontague of Chembiomed Ltd., Edmonton, Alberta, Canada its active site was shown to be αFUC(1-2) βGal(1-3) βGal. H5 is a 26KD antigen. It was expressed on monocytes as well as platelets. It was expressed by CFU-GM. H229 is a 62KD platelet antigen. It was not expressed by CFU-GM or BFU-E. mAb H4, H5, H124 and H229 were reactive with megakaryocytes but not with immature or mature myeloid cells. Table 6 and 7 summarize these data. Two manuscripts characterizing these five monoclonals in detail are provided as Appendix B and Appendix C, this report. Although these five monoclonals were well characterized, they were considered not novel.

Table 6. Expression of H229, H124, H4, H5 and K15 antigens by bone marrow cells.

	Monoclonal Antibody				
Cell Type	H229	H124	H4	H5	K15
Megakaryocyte	+	+	+	+	–
Myeloblasts	–	–	–	+	++
Promyeloblast	–	–	–	–	+
Myelocyte	–	–	–	–	–
Metamyelocyate	–	–	–	–	–
Neutrophile	–	–	–	–	–
Eosinophile	–	–	–	+	+
Erythroid - blast	–	+	–	–	–
Erythroid - nucleated	–	+	–	–	–
Lymphocytes - resting	–	±	–	–	+
Lymphocytes - activated					
Monocyte	–	–	+	+	+

The antibodies listed in Table 2 were used to stain rhesus monkey marrow cells which were depleted of T cells or mature cells reactive with SBA. Except for H5 which stains rhesus marrow cells significantly, none of them were reactive. This panel of antibodies lacks cross-reactivity, and a more direct approach to make monoclonal antibodies to rhesus marrow cells was used.

Table 7. Expression of antigen H229, H124, H4, H5 and K15 by BFU-E and CFU-GM progenitors.

Bone Marrow All Types	BFU-E/10^5 Cells Plated I	BFU-E/10^5 Cells Plated II	CFU-GM/10^5 Cells Plated I	CFU-GM/10^5 Cells Plated II
Unseparated	275±16[a]	358±41	77±15	117±35
$H229^+$	53±19	36±7	28±9	12±11
$H229^-$	340±58	452±29	78±19	132±19
HLA-DR^+	522±132	1079±62	120±7	343±11
HLA-DR^-	1±1	3±1	2±1	3±2
Unseparated	358±41	459−27	117±35	96±10
$H124^+$	1077±75	1720±369	24±8	24±3
$H124^-$	53±27	1±1	122±50	118±27
HLA-DR^+	1079±62	1493±98	343±11	461±127
HLA-DR^-	3±1	9±8	3±2	5±4
Unseparated	242±12	358±41	76±6	117±35
$H4^+$	28±5	66±10	17±5	17±12
$H4^-$	327±24	444±71	115±22	153±12
HLA-DR^+	303±24	1079±62	142±19	343±11
HLA-DR^-	23±4	3±1	2±1	3±2
Unseparated	459±27	380±23	96±10	77±12
$H5^+$	60±22	28±12	296±67	90±7
$H5^-$	453±22	383±22	22±17	22±3
HLA-DR^+	1493±98	1396±246	461±127	191±39
HLA-DR^-	9±8	3±5	5±4	1±1
Untreated	459±27	--[b]	96±10	--
$K15^+$	1425±88	--	311±85	--
$K15^-$	10±114	--	15±12	--
HLA-DR^+	1493±98	--	461±127	--
HLA-DR^-	3±8	--	5±4	--

[a]Data were presented as the mean ± standard deviation of quadruplicate

[b]Not done

2.3 USE MONOCLONALANTIBODIESAND IMMUNE ROSETTE DEPLETION TO OBTAIN A STEM CELL ENRICHED POPULATION AND ISOLATION OF THE STEM CELLS.

2.3.1 METHODS.

Five fusions were performed with either unseparated bone marrow or SBA-bone marrow cells as immunogens. In general, we used BC_3F_1 mice of ten to twelve weeks of age. Immunization was with $2x10^7$ cells per mouse with alum as adjuvant by the i.p. route. Three weeks later, $2x10^7$ cells in saline was used for i.p. injection. Three days later, spleen cells were fused with SP2/0 cells and hybridomas were selected in HAT. In general, 10 plates of 96 micro wells per plate were set up for each fusion and two mice were used for each antigen. Over 1200 hybridoma supernatants were screened. The supernatants were screened on bone

marrow and peripheral blood from four monkeys by indirect immunofluorescence with an Epic V instrument (Coulter, Hialeah, Florida). By light scattering analysis, both peripheral blood and bone marrow cells were resolved into two populations. In the case of peripheral blood mononuclear cells, they can be resolved into lymphocyte and monocyte fractions by forward angle and 90^{o} angle light scattering analysis. In the case of bone marrow cells, two populations were arbitrarily termed "small and large". The "large cells" contained mature as well as immature myeloid cells which were identified by anti-myeloid antibodies in the case of human marrow.

2.3.2 RESULTS.

Approximately 10% of the cells expressed HLA-DR. The "small" cell population contained lymphoid cells and cells express nonlymphoid markers. In this analysis, similar cellular light scattering patterns were assumed regarding rhesus bone marrow cells. Table VIII summarizes several monoclonal antibodies of interest. T11 which is now termed identified as CD2 in man is a T cell marker. However, it can be utilized as a lymphocyte marker as well. Thus, in the monocyte fraction only 3.6% of the cells stained for CD2. Similarly, there was only 18% T cell contamination in the small cells of the bone marrow. On the basis of staining, mAb SBA-73, BM25, G28, G65 and G139 were selected for further studies to determine whether they were useful in the identification of stem cells and their enrichment.

Table 8. Reactivities of monoclonal antibodies against bone marrow and peripheral blood mononuclear cells

	Bone Marrow		Peripheral Blood Mononuclear	
Monoclonal Antibodies	Small[a]	Large	Lymphocytes	Monocytes
Control	0.6[b]	4.9	0.9	4.0
T11	18.1	5.2	68	3.6
Anti-DR	not done		30	28
SBA-73	16.4	2.0	1.2	4.6
SBA-24	15.0	2.0	6.6	15
G139	25.4	100	1.2	6.2
G28	14.5	100	85	100
BM25	6.4	100	7.0	100
G65	2.0	100	1.6	100
G85	30.4	1.2	48.0	100
G34	59.02	100,weak	100	100

[a]The bone marrow cells after Ficoll/Hypaque separation can be resolved into two populations by light scattering.

[b]% positive cells

Table 9. Colony forming units are SBA⁻73, BM25, G65, G28 and G139 reactive cells

Cell Population	Experiment 1 (no./10^5 Cell Plates)			Experiment 2 (no./10^5 Cell Plates)	
	CFU-GM	BFU-E	CFU-GEMM	CFU-GM	BFU-E
SBA⁻73 reactive	140,160	60,50	10,20	30,30	30,10
SBA⁻73 nonreactive	5,0	0,0	1,0	2,4	0,0
BM25 reactive	0	0	0	--[a]	--
BM25 nonreactive	43	60	155	--	--
G28 reactive	--	--	--	30,40	10,20
G28 nonreactive	--	--	--	30,90,40,80	60,40,30,60
G65 reactive	--	--	--	10,40,20	30,30,30
G65 nonreactive	--	--	--	150,130	60,110
G139 reactive	--	--	--	60,50,70,50	30,30,30,20
G139 nonreactive	--	--	--	160,90,170, 180	40,40,10,70

[a]Not done

Table 9 shows that mAb BM25 would be useful for the negative selection of bone marrow cells enriched for stem cells. mAb SBA⁻73 was a candidate specific for stem cells. BM25 and SBA⁻73 were cloned either twice or three times on soft agar. The hybridoma was considered stable when all the clones were positive for the cell populations as the original supernatant. Regarding mAb SBA⁻73, two further experiments were done. One was confirmatory while the other showed CFU to be in the SBA⁻73 negative fraction. With additional four monkeys, CFU-c was assayed. Only one out of the four showed CFU predominantly in the SBA⁻73 reactive cells. Thus, bone marrow from only two out of six additional monkeys tested confirmed the results listed in Table 9. In addition, in the monkeys in which SBA⁻73 reactive bone marrow did not contain CFU progenitor cells, CFU were present in larger numbers in the SBA⁻73 nonreactive fraction than in the unseparated bone marrow cells. The explanation for these results is not readily apparent. It might be due to the difficulty in isolating small numbers of reactive cells. It is also possible that SBA⁻73 antigen is polymorphic. mAb BM25 was found to be consistantly nonreactive with CFU progenitor cells. Immune rosette depletion with mAb BM25 would deplete approximately 50% of the cells. The nonreactive cells were enriched 6-8 folds. Thus, we have not been able to identify stem cell antigens by monoclonal antibodies generated. However, a stable monoclonal antibody BM25 (IgG_1) was established and would be useful for the negative selection of CFU progenitors.

2.4 MAB BM25 NONREACTIVE BONE MARROW COULD RECONSTITUTE BONE MARROW CELLS IN BOTH ALLOGENEIC AND AUTOLOGOUS HOST.

While Dr. N. Gengozian was in Oklahoma, two allogeneic bone marrow transplantations were done with bone marrow cells depleted of T cells with a monoclonal antibody (T64) to the E rosette receptor and of mAb BM25 reactive cells. Both irradiated recipients showed evidence of bone marrow regeneration of donor origin. In one case, long-term survival was established, there was gradual regression of donor cells and the return of residual radiation resistant host bone marrow cells. Autologous bone marrow transplantation was also carried out by him at the University of South Florida. mAb BM25 nonreactive cells were able to reconstitute autologously. These results indicate BM25 negative bone marrow cells function in vivo.

2.5 MONOCLONAL ANTIBODIES AGAINST RHESUS PERIPHERAL BLOOD LYMPHOCYTES.

We have established monoclonal antibodies to rhesus leukocytes. These are T64 and T199 which block E rosette formation by rhesus T cells. T35 and T60 are equivalent of OKT8 and are reactive with suppressor/cytotoxic T cells. T145 is a pan B cell marker for rhesus. T64 has been used to deplete T cells for allogeneic bone marrow transplantation. It is an IgM antibody. Thus, this monoclonal antibody is efficient to deplete mature T cells from the donor marrow.

2.6 STEM CELL GROWTH FACTORS.

As stated above, the PHA-LCM from human peripheral mononuclear cells was able to support rhesus CFU-GEMM. Depletion of T8 cells did not improve the potencies of the PHA-LCM. It was also found that rhesus peripheral mononuclear cells were not superior in comparison with human cells.

2.7 HUMAN T-T HYBRIDOMA TECHNOLOGY.

It was planned to use human T-T hybridoma technology to demonstrate newer stem cell growth factor. However, technical difficulties were encountered. Before we started T-T fusion, the parental lines were tested for mycoplasma and they were found to be contaminated. Attempts were made to obtain mycoplasma free cell lines by culturing cell lines with murine peritoneal exudate cells and licomycine. Initially Molt-4 and CEM were thought to be free of mycoplasma. After further culture without licomycine, they were positive for mycoplasma. We then used the BM Bycline from Boehringer-Mannheim. After three cycles of treatment of 6 weeks, we obtained 13 T leukemia cell lines free of mycoplasma infection. They were free of mycoplasma even after they were grown in Bycline free medium. Several of the lines were CD3 negative. They were JRT3T3.5.6, SKW3, Molt-3, KE-4 and M-KE37.3.7. Some of these cell lines were used as parental partners to fuse with PHA stimulated human T cells. Molt-3 and the Jurkat mutant JRT3T3.5.6 were found to generate T-T hybrids by polyethylene glycol induced fusion and selection for CD3 positive cells which would include nonfused PHA blasts and T-T hybrids. Evidence of fusion between T blasts and leukemic cells was obtained by HLA typing with a penal of anti-HLA monoclonal antibodies from Genetic System Incorporated (Seattle, WA). With CD3 as a marker, the hybridomas were not stable with continuing loss of CD3 expression. Our Molt-3 cell line lacks CD4. We used it as a fusion partner and selected CD4 positive cells by cell sorting. The sorted cells were >99% positive for CD4. The $CD4^+$ cells were then cloned immediately on soft agar and the clones were analyzed for CD4 expression. This procedure took approximately 6 weeks to

have sufficient cells for analysis. With more than 200 clones studied, none of them retained CD4. Thus, it is concluded that the human T-T hybridoma system is very unstable. Because of these technical difficulties, we did not attempt to determine whether the T-T hybridomas had growth promoting activity.

SECTION 3

CONCLUSIONS

The rhesus monkey is an excellent model for humans in many systems. In the case of bone marrow transplantation, it has provided useful information. In the present work, attempts were made to identify stem cells by monoclonal antibodies and use human T-T hybridomas to reveal newer stem cell growth promoting activity. Limited success in the first task was accomplished. One stable antibody, i.e., BM25, is capable of negatively enriching for CFU progenitor cell activity by 6-8 fold and the marrow cells after depletion were functional in vivo in autologous and allogeneic irradiated host. Antibody SBA-73 is reactive with a small number of bone marrow cells. It may be expressed by hematopoietic stem cells. A definitive conclusion cannot be drawn because bone marrow from approximately half of the monkeys did not demonstrate CFU progenitors in the positively selected small population. The rhesus monkeys are difficult animals to obtain bone marrow repeatedly for analysis. This presents a disadvantage for the model.

Technical difficulties, i.e., instability of the T-T hybridomas and mycoplasma contamination, made the system not suitable for the identification of stem cell growth activity. Recently, recombinant DNA technology has produced the multipotential stem cell growth factor (IL-3) as well as differentiation factors such as CSF-1. This has proven to be a superior approach.

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APPENDIX A

MONOCLONAL ANTIBODIES AGAINST HUMAN MYELOMONOCYTIC CELLS: DETECTION OF CERTAIN LINEAGE-SPECIFIC ANTIGENS ON CFU-GM PROGENITOR CELLS[1]

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A series of monoclonal antibodies (mAb) were raised against nonlymphoid leukemic cell lines. Three of them have been characterized in detail. mAb H8 (IgG2), mAB U2 (IgG1), and mAb ML143 (IgM) were established with HEL, an erythroleukemia cell line, U937, a monocytoid (histiocytic) line, and ML-1, a myeloid cell line as immunogen, respectively. A 65 to 75 KD polypeptide was precipitated from monocytes by mAb H8, a 160 KD protein from monocytes by mAb U2, and two broad bands in the regions of 150 and 195 KD from granulocytes by mAb ML143. All three mAb stained peripheral blood monocytes and granulocytes, but not lymphocytes, platelets, and erythrocytes. The mAb reacted with immature myeloid cells in bone marrow, ranging from myeloblasts to mature myelomonocytic cells. They also were reactive with various nonlymphoid cell lines and leukemia of myelomonocytic origin. They did not react with B cell lines and B cell CLL cells. By complement-mediated cytolysis and/or an immune rosette method, antigens H8 and U2 were found to be expressed on the vast majority of CFU-GM (14 days) progenitors but not on BFU-E. Antigen ML143 was not expressed by either progenitor. Furthermore, ML143 antigen was found on T leukemia cell lines, a subpopulation of mitogen-activated T cells, and certain non-T/non-B ALL cells. This reactivity was not found with mAb H8 and U2. The relationship between these mAb and those reported are discussed. The possibility of using these mAb to obtain a markedly enriched CFU-GM progenitor population is also raised.

Monoclonal antibodies (mAb)[4] have been established to identify antigens expressed by human myelomonocytic cells and their precursors (1–29). Some of those have been shown to react with committed myeloid progenitors (CFU-GM).[4] In attempts to generate mAb reactive with hematopoietic progenitors, we used nonlymphoid leukemia cell lines as immunogens. among the antibodies reactive with myelomonocytic cells, three (mAb H8, U2, and ML143) have been characterized in detail. All of them stained mature and immature myelomonocytic cells. mAb H8 and U2 were shown to react with CFU-GM progenitors. They did not react with committed erythroid progenitors (BFU-E).[4] mAb ML143 did not react with either committed progenitor. It did stain a subpopulation of mitogen-activated T cells.

MATERIALS AND METHODS

Generation of mAb. Three cell lines (HEL (30), an erythroleukemia cell line kindly provided by Dr. P. Martin of the Fred Hutchinson Cancer Research Center, Seattle, WA; U937, a monocytoid-histiocytic cell line; and ML-1, a myeloid leukemia cell line) were used as immunogens. Ten-week-old BC_3F_1 female mice were injected i.p. with 2×10^7 cells with 4 mg alum as adjuvant. A second immunization was done 3 wk later with 2×10^7 cells in phosphate-buffered saline (PBS) i.p. Three days later, spleen cells were fused with SP2/0 Ag14 tumor cells with PEG 1000. Hybridomas were selected in HAT medium; their supernatants were screened by indirect immunofluorescence. The desired hybridomas were cloned on agarose with fibroblasts as feeders. Details of these procedures have been described (31).

Other mAb used in the present studies were mAb Josh 524, mAb M3, mAb TE, mAb H8, Leu-10, and B1. The first four antibodies were generated in our laboratory. mAb Josh 524 (IgG1) is specific for HLA-DR. It precipitated a 29/34 kilodalton (KD) bimolecular complex. mAb M3 (IgG1) is specific for μ-chain. mAb TE (IgG2) blocked rosette formation between T cells and sheep erythrocytes (RBC). It precipitated a 55 KD glycoprotein; it is equivalent to anti-T11 mAb. Leu-10, an anti-HLA-DC antibody, was obtained from Becton Dickinson (Mountain View, CA). B1, a B cell-specific mAb, was obtained from Coulter Immunology (Hialeah, FL). As appropriate control mAb, SS1 (IgG2a, anti-sheep RBC), NS4.1 (IgM, anti-sheep RBC), and HDP-1 (IgG1, anti-DNP) were used. These antibodies were kindly provided by Dr. J. Davie of Washington University (St. Louis, MO).

Cell preparation. Defibrinated blood from normal volunteers was used as a source of peripheral blood mononuclear cells (PBMC). PBMC were separated as described (32). Monocytes were separated by Percoll continuous gradient centrifugation (33). Granulocytes were isolated from the peripheral blood as the pellet fraction after Ficoll-Hypaque density gradient centrifugation. Contaminating RBC were removed by a 60%, 65%, and 70% Percoll discontinuous density gradient centrifugation. The purity of the granulocyte fraction was more than 94% as determined by Wright-Giemsa staining. Platelets were isolated from platelet-rich plasma and RBC from the pellets of Ficoll-Hypaque gradients of peripheral blood cells.

Leukemia cells of different lineages were isolated from the peripheral blood of the affected patients by Ficoll-Hypaque gradient centrifugation. They were cryopreserved and were recovered before staining.

Cell lines. In addition to HEL, U937, and ML-1 cell lines, KG-1, a

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[4] Abbreviations used in this paper: CFU-GM, colony-forming units (granulocyte/monocyte); BFU-E, burst-forming units (erythroid); KD, kilodalton; PBMC, peripheral blood mononuclear cells; mAb, monoclonal antibody.

myeloblastic leukemia cell line. K562. an erythroid/myeloid line from a patient with CML in blastic crisis. and HL-60. a promyelocytic cell line. were used as nonlymphoid lines. For T leukemia cell lines. RPMI 8402. Mt-1. Molt-4. Jurkat. and CEM were used. As B lymphoblastoid cell lines. Raji. Daudi. Josh 7. a pre-B cell line (34). 866P (IgG). 32a1. (IgA-producing). and SeD (35) were used. All cell lines were cultured in RPMI 1640 supplemented with 10% FBS.

Bone marrow cells and bone marrow cultures. Bone marrow cells were obtained from normal volunteers. They were separated by Ficoll-Hypaque density centrifugation to remove RBC and mature granulocytes. Separated bone marrow cells were subjected to an immune rosette method (36) to obtain cells reactive with the mAb of interest. or they were subjected to a complement (C)-mediated cytolysis procedure to deplete the reactive cells. For the C-mediated killing procedure. 0.5 ml of 2×10^7/ml of bone marrow cells were incubated with 0.5 ml of hybridoma culture supernatant for 30 min at 37°C. One milliliter of baby rabbit C (Pel-Freez Biologicals. Rogers. AR) was added and the mixture was incubated for 45 min at room temperature. Dead cells were removed by centrifugation on a Ficoll-Hypaque gradient. Although one-cycle killing was employed in these experiments. the recovered cells contained less than 0.1% of the reactive cells by the indirect immunofluorescent assay with the Epics V flow cytometer. Thus. no attempts were carried out to repeat the killing by a second-cycle treatment. Bone marrow cells were cultured in quadruplicates in Iscove's modified Dulbecco's medium containing 0.9% methylcellulose. 10% conditioned medium from human peripheral blood leukocytes stimulated with phytohemagglutinin. and 30% FBS. One unit of erythropoietin (sheep fraction III: Connaught. Toronto. Ontario. Canada) was added to each plate. This procedure was essentially as described by Messner et al. (37). BFU-E were scored as hemoglobin-containing single or multiple colonies of greater than 64 cells on the 14th day. This culture also allowed CFU-GM colony formation. CFU-GM were scored as colonies of greater than 40 cells on the 14th day.

Immunofluorescence studies. Cells (0.05 to 1×10^6) were first incubated with hybridoma culture supernatants for 20 min. After three washings with 0.01 M PBS with 1% bovine plasma albumin. fluorescein-labeled $F(ab')_2$ anti-mouse Ig was added and a 20-min incubation at 4°C was performed. After three washings. cells were analyzed with a Coulter Epics V flow cytometer (Coulter Electronics. Hialeah. FL). Integrated fluorescence of the population gated by forward angle light scatter and right angle light scatter was measured and 10.000 cells were analyzed.

Iodination and immunoprecipitation. Cells were iodinated in suspension by the method of Hubbard and Cohn (38). In brief. 2×10^7 monocytes were incubated with 1 mCi/ml Na ^{125}I. 50 mU/ml type V glucose oxidase (Sigma Chemical Co.. St. Louis. MO). and 10 μg/ml lactoperoxidase (Calbiochem-Behring Corp.. San Diego. CA) for 15 min on ice. The reaction was terminated by aspirating the supernatant and by repeated washings with RPMI 1640. After iodination. immunoprecipitation. gel electrolysis. and autoradiography were performed as described (39).

RESULTS

Characterization of mAb H8. U2. and ML143. mAb H8. U2. and ML143 were established with HEL. U937. and ML-1 as immunogen. respectively. By ELISA. mAb H8 was typed to be IgG2; mAb U2. IgG1; and mAb ML143. IgM. Both mAb H8 and ML143 fixed C; mAb U2 did not.

mAb H8 precipitated a molecule with a broad band between 65 and 75 KD from ^{125}I-labeled monocytes (Figure 1. lane 1). A similar molecule was precipitated from HEL. the immunogen. mAb U2 precipitated a glycoprotein of 160 KD from ^{125}I-labeled monocytes (Figure 1. lane 3) and U937. the immunogen. These m.w. were estimated from 9% SDS gels in reduced conditions. mAb ML143 precipitated two broad bands at 150 and 195 KD from iodinated granulocytes under reduced conditions (Figure 1. lane 5). Under nonreduced conditions. similar diffuse bands were seen. With ^{125}I-labeled monocytes or ML-1 cells. these two bands were less distinct.

Peripheral blood cell distribution of antigens H8. U2. and ML143. The cellular distribution of the reactive antigens by mAb H8. U2. and ML143 was analyzed by immunofluorescence with an Epics V flow cytometer.

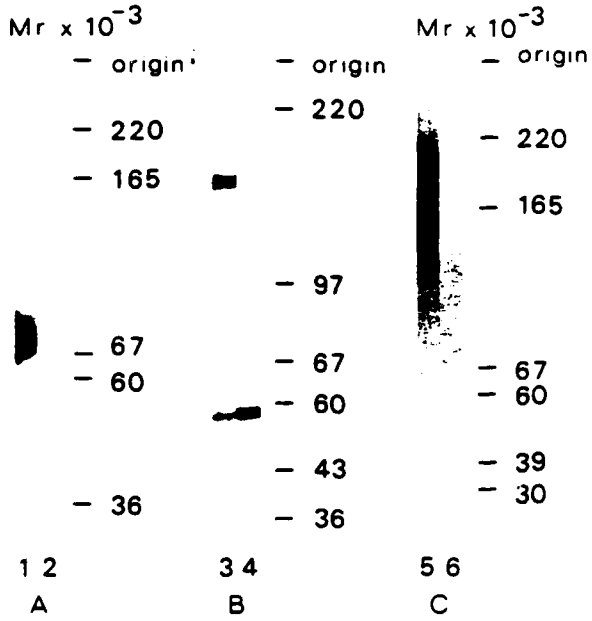

Figure A-1. A 65 to 75 KD glycoprotein was precipitated by mAb H8 from ^{125}I-labeled monocytes (lane 1). No band was seen with mAb SS1 (IgG2a. anti-sheep RBC) as a control antibody (lane 2). A 160 KD glycoprotein was precipitated by mAb U2 from ^{125}I-labeled monocytes (lane 3). Both mAb U2 and control mAb HDP-1 (IgG1. anti-DNP) brought down a nonspecific 58 KD band. mAb ML143 precipitated two broad bands at 150 KD and 195 KD from ^{125}I-labeled granulocytes. Control IgM antibody NS4.1 (anti-sheep RBC) did not bring down any discernible band.

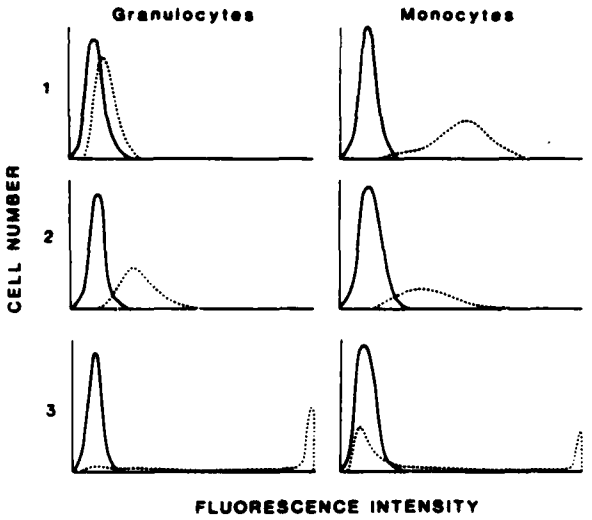

Figure A-2. Fluorocytometric analysis of staining patterns of peripheral blood granulocytes and monocytes with mAb H8 (panel 1). mAb U2 (panel 2). and mAb M143 (panel 3). ——. control mAb; ·····. mAb under investigation.

From peripheral blood. monocytes. granulocytes. and lymphocytes were isolated by a series of Ficoll-Hypaque and Percoll gradients. Platelets and RBC were also isolated. These cell preparations contained greater than 90% of the desired cells. The cells were first gated by forward angle and 90-degree light scattering. Integrated green fluorescence was measured. The results are shown in Figure 2 and Table I. mAb H8 stained monocytes brightly. but stained granulocytes weakly. The staining

TABLE A-1

Staining of peripheral blood cells by mAb H8, U2, and ML143

Cells	mAb				
	H8 (%)	U2 (%)	ML143 (%)	TE (sheep RBC receptor) (%)	Josh 524 (HLA-DR) (%)
Monocytes	96.7 ± 1.6[a]	95.2 ± 1.6	63.7 ± 15.8	3.0 ± 1.8	92.6 ± 2.8
Granulocytes	>30[b]	>90	96.3 ± 3.3	<1.0	<1.0
Lymphocytes	<1.0	<1.0	<1.0	87.4 ± 2.9	11.6 ± 3.3
RBC	<1.0	<1.0	<1.0	<1.0	<1.0
Platelets	<1.0	<1.0	<1.0	<1.0	<1.0

[a] This represents the mean and the standard deviation of determinations of samples from six individuals. Cells were stained with the hybridoma supernatants and were washed. They were then stained with fluorescein-conjugated goat anti-mouse Ig and were analyzed by an Epics V instrument.
[b] See text.

of granulocytes gave a peak shift from the control antibody-staining pattern. Although we calculated that approximately 30% of the cells were outside the control curve, the whole granulocyte population was weakly reactive. mAb U2 stained both granulocytes and monocytes strongly. mAb ML143 reacted with the majority of the granulocytes. A subpopulation of monocytes were strongly stained, but a distinct nonreactive population was also evident (Figure 2, panel 3). All three mAb failed to react with lymphocytes, RBC, and platelets. mAb TE and Josh 524 were included in Table I to characterize the isolated cell populations.

Hematopoietic cell lines and leukemias. Antigen expression by various human hematopoietic cell lines are shown in Table II. mAb H8 reacted with all six nonlymphoid lines. mAb U2 stained KG-1, HL-60, U937, and HEL. Antigen U2 was not expressed by cell lines ML-1 and K562. mAb ML143 stained five of the six nonlymphoid lines, with HEL being the nonreactive cell line. Both mAb H8 and U2 were found to be nonreactive with T and B lymphoid lines. Despite its nonreactivity to lymphocytes, mAb M143 stained all five T leukemia cell lines. The least reactive T cell line was Molt 4, with 17.8% positive cells.

Leukemic cells of both lymphoid and myeloid origin were used to further study the expression of these antigens (Table III). Seven cases of acute myeloid and monomyelocytic leukemias were available. All three mAb were reactive with the leukemic blasts in varying degrees in most cases. mAb ML143 reacted least well. One case of AML was clearly nonreactive. It should be noted that antigen ML143 was expressed on some cells of common acute lymphocytic leukemia. Although not included in Table III, all three antibodies were reactive with two cases of CML but not with cells of one case of T-ALL, one case of T-CLL, and six cases of B-CLL. These results are in general agreement with those on cell lines.

Antigen ML143 expression on activated T cells. Because of the reactivity of mAb ML143 with T leukemia cell lines, its reactivity with activated T cells was investigated. Isolated T cells were activated with Con A at 5 μg/ml for 3 days. The T blasts were stained. As shown in Table IV, mAb ML143 stained 21 to 63% of the blasts in four separate experiments. In contrast, the other two mAb did not stain. All three mAb did not react with activated B cells in three experiments, nor did they react with three thymocyte preparations.

Antigenic expression on bone marrow cells. The reactivity of bone marrow cells to mAb H8, U2, and ML143 was studied by two methods. Bone marrow cells were separated into reactive and nonreactive populations by an immune rosette method. In the case of mAb H8, bone marrow cells were incubated with the supernatant of H8 hybridoma. After several washings with PBS, they were rosetted with human RBC coated with goat anti-mouse Ig. The $H8^+$ population was separated from the nonro-

TABLE A-2

Reactivity of hematopoietic cell lines of diverse lineages to mAb H8, U2, and ML143

Cell Line	Cell Type	mAb		
		H8 (%)	U2 (%)	ML143 (%)
ML-1	myeloblastic	99.4 ± 0.5[a]	<5	97.1 ± 2.7
KG-1	myeloblastic	>80	89.4 ± 7.6	79.7 ± 11.7
HL-60	promyelocytic	>80	95.4 ± 3.4	94.5 ± 2.2
U937	monocytic/histiocytic	>80	97.0 ± 2.9	96.2 ± 4.5
HEL	erythroleukemia	97.7 ± 0.9	95.2 ± 3.9	<5
K562	myeloid/erythroid	>80	<5	96.7 ± 1.9
MT-1	T cell leukemia	<5	<5	73.2 ± 11.9
Molt-4	T cell leukemia	<5	<5	17.8 ± 4.2
Jurkat	T cell leukemia	<5	<5	71.5 ± 5.3
CEM	T cell leukemia	<5	<5	63.9 ± 5.6
RPMI 8402	T cell leukemia	<5	<5	66.4 ± 7.5
Raji	B lymphoid	<5	<5	<5
Daudi	B lymphoid	<5	<5	<5
Josh 7	B lymphoid	<5	<5	<5
8866P	B lymphoid	<5	<5	<5
32al	B lymphoid	<5	<5	<5
SeD	B lymphoid	<5	<5	<5

[a] Data are presented as mean ± SD of three separate experiments. When there were substantial overlaps between the control and experimental data, without clearly delineating the stained population, >80% was used to indicate that the majority of the cells were stained. Because of the occasional light stained cells in control samples, <5% staining is considered negative.

TABLE A-3

Reactivity of anti-monocyte-granulocyte mAb with acute myelocytic and myelomonocytic and lymphocytic leukemia

Leukemia Type	mAb				
	H8 (%)	U2 (%)	ML143 (%)	Josh 524(HLA-DR) (%)	Leu10(HLA-DC) (%)
Acute myelocytic					
1	94[a]	83	18	58	<1
2	90	56	7	90	<1
3	53	10	44	79	<1
4	27	51	<1	81	<1
5	ND[b]	49	14	90	56
6	66	46	20	65	78
Acute monomyelocytic					
1	41	81	65	66	23
Acute lymphocytic (common ALL)					
1	<1	<1	77	90	<1
2	<1	<1	6	95	10

[a] Percent positive cells stained by the mAb.
[b] ND, not done.

TABLE A-4

Antigen ML143 is expressed on Con A-activated T cells[a]

Cell Type	mAb		
	H8 (%)	U2 (%)	ML143 (%)
Con A-activated T cells	<2	<2	21-82
Activated B cells	<2	<2	<2
Thymocytes	<2	<2	<2

[a] T cells were activated by 5 μg Con A/ml. B cells were activated by anti-μ antibodies and BSF-containing conditioned medium. Blasts at day 3 cultures were stained. Thymocytes from three preparations were studied.

TABLE A-5

Bone marrow cells reactive with anti-monocyte-granulocyte antibodies[a]

Cell Type	mAb		
	H8	U2	ML143
Myeloid			
Myeloblasts	+	+	±
Promyelocytes	+	+	+
Myelocytes	+	+	+
Metamyelocytes	+	+	+
Neutrophils	+	+	+
Eosinophils	+	+	+
Monocytes	+	+	+
Erythroid blasts	−	−	−
Megakaryocytes	−	−	−

[a] Bone marrow cells were separated into two populations by an immune rosette method with the mAb being investigated. Both positive and negative populations were examined morphologically with Wright-Giemsa stain. "+" indicates that >95% of the cells formed rosettes; "−", less than 5% of cells were reactive; and "±", 15 to 25% were positive.

sette-forming H8⁻ population by centrifugation on a Ficoll-Hypaque gradient. The attached RBC were lysed. H8⁺ and H8⁻ populations were fixed and were stained with Wright-Giemsa stain. As shown in Table V, antigen H8 was expressed by cells of the myelomonocytic lineage but not by cells of erythroid lineage, megakaryocytes, and platelets. Similar results were obtained with mAb U2. In the case of mAb ML143, a small number of myeloblasts (15 to 25%) was seen in the ML143⁻ population.

The staining of bone marrow cells was also analyzed by flow fluorocytometry. The bone marrow cells were resolved into two populations by light-scattering analysis with forward and 90-degree light scattering (Figure 3). One population with more light scattering is arbitrarily termed the "large cell" population, while that with less light scattering is referred to as the "small cells." These two populations were analyzed with a panel of mAb. It is apparent that the small cell population contained lymphocytes of both T and B cell lineages and most of the

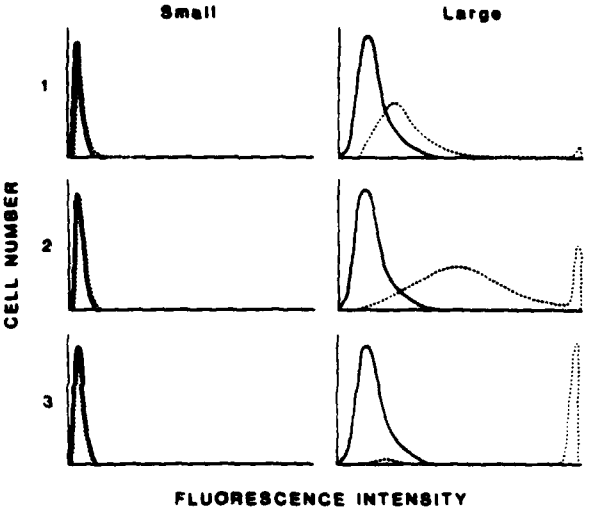

Figure A-3. Staining patterns of bone marrow cells by mAb H8 (panel 1), mAb U2 (panel 2), and mAb ML143 (panel 3). The bone marrow cells were gated into two populations by forward angle vs 90-degree light scattering. The cells with more light-scattering characteristics were arbitrarily termed "large" cells. The other population was called "small" cells. ——, control mAb; ·····, mAb under investigation.

TABLE A-6

Antigenic expression by two populations of bone marrow cells resolved by light-scattering analysis

mAb	Unfractionated Bone Marrow	
	large	small
Ab 23.1 (SRBC receptor)	<1.0%	41.2
Ab Josh 524 (HLA-DR)	10.1	27.0
Leu-10 (HLA-DC)	<1.0	17.6
Anti-μ	<1.0	8.5
B1	<1.0	9.5
H8	>80	8.7
U2	74.7	5.9
ML143	75.5	1.8

HLA-DR⁺ cells (Table VI). Undoubtedly, some of the lymphocytes were due to contaminating blood cells. All three mAb (H8, U2, and ML143) stained the majority of the cells in the large cell population. A small number of cells in the small cell population were reactive with these mAb. It remains to be determined whether some of these reactive cells in this population are CFU-GM progenitors. Two additional bone marrow samples were analyzed and showed similar results.

Presence of antigens H8 and U2 but not ML143 on CFU-GM progenitors. mAb H8 and ML143 were found to fix C. By using C-mediated cytolysis, antigen H8 was found to be present on CFU-GM but not on BFU-E (Table VII). In two experiments, the treatment of bone marrow cells by mAb H8 in the presence of C eliminated over 90% of CFU-GM progenitors. This treatment did not change the number of BFU-E substantially. A similar treatment with mAb ML143 and C failed to inhibit either BFU-E or CFU-GM.

These findings were confirmed by an alternative cell separation method. By our immune rosette method, the rosetting and nonrosetting cells were examined before lysis. The positive populations contained 90 to 95% desired cells; the nonrosetting population contained less than 5% rosetting cells. The yield of this immune rosette method was 40 to 60% of the expected cell numbers in these populations. As shown in Table VIII, $H8^+$ bone marrow cells isolated by an immune rosette method contained more than 80% of CFU-GM. In contrast, $H8^-$ cells contained the vast majority of BFU-E. In the case of mAb ML143, both BFU-E and CFU-GM progenitors were largely contained in the $ML143^-$ population. In addition, antigen U2 was found to be expressed by CFU-GM progenitors but not by BFU-E progenitors. This is similar to the distribution of antigen H8. In these experiments, mAb Josh 524 (anti-HLA-DR) was utilized as a control antibody. In all experiments, CFU-GM and BFU-E progenitors were shown to be $HLA\text{-}DR^+$.

TABLE A-7

Expression of antigens H8 and ML143 on BFU-E and CFU-GM as determined by C-mediated lysis

Expt.	% Dead Cells	No. Colonies/10^5 Cells Plated	
		BFU-E	CFU-GM
1. Untreated	0.9	88.5 ± 14.0[a]	45.8 ± 7.0
C alone	1.1	105.8 ± 3.5	52.0 ± 6.7
mAb H8 + C	22.3	84.0 ± 14.8	4.7 ± 0.5
2. Untreated	0.8	59.0 ± 11.0	70.7 ± 14.6
C alone	0.9	49.0 ± 13.0	60.0 ± 7.1
mAb H8 + C	36.1	74.0 ± 13.1	0
3. Untreated	2.9	88.0 ± 14.0	46.0 ± 7.0
C alone	2.8	106.0 ± 4.0	52.0 ± 7.1
mAb ML143	23.6	80.2 ± 20.3	50.2 ± 15.7
4. Untreated	1.8	242.5 ± 12.2	76.2 ± 6.3
C alone	2.3	224.1 ± 35.4	83.4 ± 5.3
mAb ML143	53.9	311.0 ± 36.7	155 ± 19.5

[a] Data are presented as the mean ± standard deviation of quadruplicate plates.

TABLE A-8

Bone marrow CFU-GM progenitor cells expressed antigens H8 and U2 but not antigen ML143; none of these antigens were expressed by BFU-E progenitors

Bone Marrow Cell Type	BFU-E/10^5 Cells Plated		CFU-GM/10^5 Cells Plated	
	I	II	I	II
Unseparated	630 ± 65[a]	275 ± 16	100 ± 11	77 ± 15
$H8^+$	53 ± 5	58 ± 11	160 ± 27	111 ± 23
$H8^-$	677 ± 38	225 ± 27	28 ± 5	12 ± 1
$HLA\text{-}DR^+$	539 ± 153	522 ± 132	125 ± 2	120 ± 7
$HLA\text{-}DR^-$	3 ± 2	1 ± 1	1 ± 0	2 ± 1
Unseparated	459 ± 27	380 ± 23	96 ± 10	77 ± 12
$U2^+$	23 ± 16	26 ± 21	206 ± 54	137 ± 14
$U2^-$	441 ± 61	419 ± 58	40 ± 16	32 ± 17
$HLA\text{-}DR^+$	1493 ± 98	1396 ± 246	461 ± 127	191 ± 39
$HLA\text{-}DR^-$	9 ± 8	3 ± 5	5 ± 4	1 ± 1
Unseparated	192 ± 28	213 ± 27	65 ± 15	93 ± 19
$ML\ 143^+$	68 ± 19	55 ± 11	16 ± 13	32 ± 7
$ML\ 143^-$	283 ± 21	239 ± 37	95 ± 13	129 ± 23

[a] Data are presented as the mean ± standard deviation of quadruplicate plates.

DISCUSSION

A series of mAb were raised against nonlymphoid hematopoietic cell lines. The characteristics of three of them are detailed in this report. mAb H8, U2, and ML143 were established by using HEL, U937, and ML-1 cell lines as their respective immunogen. They have been shown to react with peripheral blood monocytes and granulocytes preferentially. Cellular distribution and immunoprecipitation analysis indicate that these three antibodies identify distinct antigens on myelomonocytic lineage cells. The results of studies involving leukemia cells, cell lines, and activated lymphocytes add further support to the concept that antigens H8 and U2 are specifically expressed by myelomonocytic cells and their precursors. These studies also reveal that ML143 antigen is also expressed by certain activated T cells. With a C-mediated cytolysis and/or a positive selection method, mAb H8 and U2 were reactive with the vast majority of CFU-GM progenitors but not with BFU-E progenitors. The possibility that a small population of CFU-GM progenitor cells did not express these antigens has not been ruled out. In contrast, mAb ML143 was not reactive with either progenitor. Thus, these three mAb have two distinct reactive patterns despite their reactivity with monocytes and granulocytes.

Many mAb have been reported to be preferentially reactive with myelomonocytic cells (1–28). It is relevant to relate our mAb to them. mAb H8 (IgG2) is reactive with a glycoprotein of 65 to 75 KD. To our knowledge, none of the reported mAb identifies such a protein with a cellular distribution of the H8 antigen. However, several reported mAb need to be discussed further. mAb Mo2 identifies a 55 KD protein. It reacts with monocytes, macrophages, and their precursors (27). Its nonreactivity to granulocytes, CFU-GM progenitors, HL-60, K562, and U937 cell lines distinguishes it from mAb H8. mAb 61D3 (19) was shown to react with 75 KD protein on monocytes. In the original report, it was stated that it did not react with granulocytes and cell line U937. However, little information is available as to its reactivity to other myelomonocytic and erythroid cell lines and CFU-GM progenitors. Based on this information, we tentatively conclude that mAb H8 and 61D3 identify different antigens. Definitive experiments involving sequential immunoprecipitation are needed to draw a firmer conclusion. mAb My906 identifies as a 72 KD molecule (28). It reacts with monocytes, AML cells, and CFU-GM progenitors. Its reactivity with BFU-E and its nonreactivity with granulocytes suggests that antigen My906 is distinct from antigen H8. Thus, it appears that mAb H8 identifies a new myelomonocytic antigen.

mAb U2 identifies a myelomonocytic antigen of 160 KD glycoprotein. This m.w. is very similar to that of antigen My7 (27). Cellular distribution for My7 and U2 is also similar. In addition, both antigens are expressed by CFU-GM progenitors. It is likely that both mAb are reactive with a similar glycoprotein.

mAb ML143 precipitated two broad bands (150 and 195 KD) of glycoproteins from ^{125}I-labeled granulocytes under reduced and nonreduced conditions. It is of considerable interest to note that these two bands were less

distinct when precipitation was carried out with either ML-1 cells or monocytes. The broadness of the band and variable results from different cell preparations add support to the thesis that mAb ML143 is reactive with an antigenic determinant attributed largely to the carbohydrate moiety on the glycoproteins. Recently, mAb T5A7, which reacts with myelomonocytic cells and a subpopulation of mitogen-activated T cells as well as late myeloid progenitors (day 7 CFU-GM), has been identified to be specific for lactosylceramide (40). It is likely that mAb ML143 and mAb T4A7 have similar specificity. It remains to be determined if mAb T5A7 will also precipitate glycoproteins from granulocytes. Studies with both antibodies will resolve the question of antigenic identity. Although mAb PM-81 (22) was shown to be nonreactive with PHA-activated T cells and CEM cell lines, its staining pattern with hematopoietic cells, cell lines, and leukemia cells was very similar to mAb ML143. Thus, it is probable that all three mAb (ML143, T5A7, and PM-81) are reactive with a similar carbohydrate determinant. It is also of considerable interest that all of them are IgM antibodies.

In addition to mAb143, 10 other mAb from two independent fusions were shown to react with myelomonocytic cells and to precipitate similar broad bands (155 to 195 KD) from ^{125}I-labeled granulocytes. They are all IgM antibodies. This suggests that ML143 antigen is a dominant antigen on ML-1 cells to which BC_3F_1 animals are responding. My-1 has been identified to be against a sugar sequence found in lactose-*N*-fucoperitose III (41). Although it is not expressed by CFU-GM progenitors, it differs from ML 143, TSA 7, and PM-81 in that mAb My-1 did not stain either leukemic T cell lines or T blasts (20). Nevertheless, all five mAb to My-1 were IgM antibodies. Relevant to this discussion are the findings that mAb such as IG10 (42) and Pro-I12 Im2 (27), which were identified to react with carbohydrate determinants on glycoproteins and glycolipids, are also IgM antibodies. It is also of considerable interest that carbohydrate determinants are expressed by hematopoietic cells at defined stages of differentiation. However, it is likely that other tissues would express these antigens. Thus, more extensive tissue distribution studies need to be carried out before they can be utilized for specific immunotherapeutic purposes.

mAb have been successfully utilized to purify progenitor cells of various hematopoietic lineages (26, 43–46). Most of them are reactive with progenitor cells of different lineages. Recently, an mAb specific for erythroid progenitors has been identified (46). A survey of the literature reveals that similar mAb to myelomonocytic progenitors (CFU-GM) have not been reported. It is possible to utilize mAb such as mAb H8 and/or mAb U2 in combination with mAb ML143 to obtain a bone marrow cell population with a high cloning efficiency for CFU-GM. This population would be useful to aid in the characterization of CFU-GM progenitors morphologically and to use as immunogens to establish mAb specific for these progenitors.

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APPENDEX B

MONOCLONAL ANTIBODY K15 DETECTS A 22 kD PROTEIN ANTIGEN ON PROGENITOR CELLS OF CFU-GM AND BFU-E[1]

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Abbreviations

CFU-GM = colony-forming units (granulocyte/monocyte); BFU-E = burst-forming units (erythroid); PBS = phosphate buffered saline; PBMC = peripheral blood mononuclear cells; mAb = monoclonal antibody; sIg = surface immunoglobulin.

ABSTRACT

Monoclonal antibodies (mAb) were raised against the human erythroid cell line K562. One of them, mAb K15, was reactive with a 22 kD polypeptide. It was shown to be reactive with monocytes, lymphocytes, eosinophilic granulocytes, thymocytes, leukemic cells of diverse hematopoietic lineages and cells from various hematopoietic cell lines. Antigen K15 was not expressed by platelet, erythrocytes, megakaryocytes, erythroblasts, neutrophilic granulocytes and myelocytes. K15 antigen was shown to be present on progenitor cells of CFU-GM and BFU-E. mAb K15 in the presence of complement eliminated CFU-GM and BFU-E progenitor cells from normal bone marrow. Positively selected K15+ cells were enriched for these progenitor cells. With a combination of mAb K15, mAb against T and B cells, cells of the monocyte-granulocyte series and an anti-HLA-DR mAb, a bone marrow cell population with approximately 10% cloning efficiency for BFU-E was obtained. The possibility of K15 antigen expressed on the multipotential progenitor cell is discussed.

INTRODUCTION

Monoclonal antibodies (mAb) have been utilized to identify and to enrich human hematopoietic progenitor cells (1-9). Some of the monoclonal antibodies are more specific for the progenitor cells while the others are less specific. Nevertheless, their usefulness has been clearly established. Recently, a monoclonal antibody K15 has been established in our laboratory. K562 cell line was used as the immunogen. This antibody stained monocytes and lymphocytes as well as progenitor cells of CFU-GM and BFU-E. Despite its broad reactivity, mAb was useful in the enrichment of BFU-E progenitor cells.

MATERIALS AND METHODS

Monoclonal antibody production

Ten week old BC_3F_1 female mice were injected intraperitoneally with 2 x 10^7 cells of K562, a cell line derived from a CML patient in blastic crisis with 4 mg alum as adjuvant. Three weeks later, 2 x 10^7 K562 cells in phosphate buffer saline (PBS) was injected i.p. Fusion of spleen cells with SP2/0 murine tumor cells was performed three days later. Hybridomas were selected in HAT medium. Supernatants were screened by indirect immunofluorescence. The desired hybridomas were cloned in soft agarose with fibroblasts as feeder cells. Details of the procedures were described elsewhere (10). mAb K15 was one of the antibodies generated by these fusions.

Other monoclonal antibodies used in the present studies are mAb Josh 524, mAb M3, mAb TE, mAb HB, mAb Leu 10 and mAb B1. The first four antibodies were generated in our laboratory. mAb Josh 524 (IgG,) is specific for HLA-DR. It precipitated a 29/34 kD bimolecular complex. mAb M3 (IgG_1) is specific for μ chain. mAb TE (IgG_2) blocked rosette formation between T cells and sheep erythrocytes. It precipitated a 55 kD glycoprotein. It is equivalent to mAb T11. mAb H8 is an IgG_2 antibody reactive with monocytes, granulocytes and progenitor cells of CFU-GM but not BFU-E (11). mAb Leu 10 is an anti-HLA-DC antibody and was obtained from Becton-Dickinson, Mountain View, CA. mAb B1, a B cell specific mAb, was obtained from Coulter, Hialeah, FL. As a control antibody, NS7 (IgG_3) specific for sheep red cells was used.

Cell preparation

Defibrinated blood from normal volunteers was used as a source of peripheral blood mononuclear cells (PBMC). PBMC were separated as described previously (12). Monocytes were separated by Percoll continuous gradient centrifugation (13). Granulocytes were isolated from the peripheral blood as the pellet fraction after Ficoll-Hypaque density gradient centrifugation. Contaminating erythrocytes were removed by centrifugation with a 60%, 65% and 70% Percoll discontinuous density gradient. The purity of the granulocyte fraction was more than 94% as determined by Wright-Giemsa staining. Platelets were isolated from platelet rich plasma and erythrocytes from the pellets of Ficoll-Hypaque gradients of peripheral blood cells.

Cell lines

The following non-lymphoid cell lines were used: ML-1, a myeloblastic leukemia cell line; U937, a histocytic lymphoma cell line and monocytoid line; KG-1, a myeloblastic leukemia cell line; K562, an erythroid-myeloid cell line from a patient CML in blastic crisis; HL-60, a promyelocytic leukemia cell line; and HEL, a erythroleukemia cell line. HEL (14) was kindly provided to us by Dr. Paul Martin of the Fred Hutchinson Cancer Research Center, Seattle, WA. For T leukemia cell lines, RPMI-8402, MT-1, Molt-4, Jurkat and CEM were used. As B lymphoblastoid cell lines, Raji, Daudi, Josh-7, a pre-B cell line (15), 8866P (IgG), $32a_1$ (IgA producing) and SeD (16) were used. All cell lines were cultured in RPMI-1640 supplemented with 10% FBS.

Bone marrow cells and bone marrow cultures

Bone marrow cells were obtained from normal volunteers. They were separated by Ficoll-Hypaque density centrifugation to remove erythrocytes and mature granulocytes. Separated bone marrow cells were subjected to an immune rosette method (11) to obtain cells reactive with the monoclonal antibody of interest or they were subjected to a complement mediated cytolysis procedure to deplete the reactive cells. For the complement mediated cytolysis, 0.5 2×10^7 bone marrow cells were incubated with 0.5 ml of hybridoma culture supernatant for 30 min at 37°C. 1.0 ml of rabbit baby complement (Pel Freez Biologicals, Rogers, AR) was added and the mixture was incubated for 45 min at room temperature. Dead cells were removed by centrifugation on a Ficoll-Hypaque gradient. Bone marrow cells were cultured in quadruplicates in Iscove's modified Dulbecco's medium containing 0.9% methylcellulose, 10% conditioned medium from human peripheral blood leukocytes stimulated with phytohemagglutinin (PHA-LCM), 30% FBS. One unit of erythropoietin sheep fraction III (Connaught, Toronto, ONT) was added to each plate. This procedure was essentially that described by Messner, et al. (18). BFU-E were scored as hemoglobin-containing single or multiple colonies of greater than 64 cells on the 14th day. This culture condition also allowed CFU-GM colony formation. CFU-GM were scored as colonies of greater than 40 cells on the 14th day.

Immunofluorescence studies

Cells ($0.05-1.0 \times 10^6$) were first incubated with hybridoma culture supernatants for 20 min. After three washings with PBS with 1% bovine plasma

albumin, fluorescein labeled $F(ab')_2$ anti-mouse Ig was added and a 20 min incubation at 4°C carried out. After three washings, cells were analyzed with a Coulter Epics V flow cytometer (Coulter Electronics, Hialeah, FL). Integrated fluorescence of the population gated by forward angle light scatter and right angle light scatter was measured and 10,000 cells were analyzed.

Iodination and immunoprecipitation

Cells were iodinated in suspension by the method of Hubbard and Cohn (19). Briefly, 2×10^7 monocytes were incubated with 1 mCi/ml Na ^{125}I, 50 mU/ml type V glucose oxidase (Sigma, St. Louis, MO), and 10 µg/ml lactoperoxidase (LPO; Calbiochem-Behring Corp., San Diego, CA) for 15 min on ice. The reaction was terminated by aspirating the supernatant and repeated washings with RPMI-1640. After iodination, immunoprecipitation, gel electrolysis and autoradiography were performed as described previously (20).

RESULTS

Characterization of mAb K15

mAb K15 was shown to be an IgG_3 antibody. It precipitated a 22 kD polypeptide from ^{125}I-labeled K562 cells (Figure 1). A similar 22 kD peptide was also precipitated from peripheral blood mononuclear cells.

Reactivity with peripheral blood cells

Lymphocytes, monocytes, granulocytes, erythrocytes and platelets were isolated from peripheral blood. These preparations contained 90-98% of the desired cells by morphological analysis with Wright-Giemsa staining. For the monocyte population, non-specific esterase staining was used. The cells were analyzed for the presence of the 22 kD antigen with mAb K15 by indirect immunofluorescence method with an Epic V instrument. The cells were gated by forward angle and 90° light scattering. Integrated green fluorescence was measured. The staining patterns for lymphocytes, monocytes and granulocytes are shown in Figure 2.

The majority of lymphocytes and monocytes were stained by mAb K15. The staining intensity of the monocytes was less than that for lymphocytes. In the case of granulocytes, the vast majority of the cells were non-reactive. However, a small percentage was positive. To identify the reactive granulocytes, isolated granulocytes were incubated with mAb K15. After extensive washings, the cells were mixed with human erythrocytes coated with goat anti-mouse Ig antibodies. After 30 min incubation, the cells were spread on a slide and air dried. The cells were stained with Wright-Giemsa stain. Only eosinophiles were found to form rosettes, indicating that the positive cells detected by flow fluoreocytometry were eosinophiles. The results of six experiments are summarized in Table 1. In addition to peripheral blood cells, three thymocyte preparations were also found to express antigen K15. Thus, mAb K15 stained lymphocytes, monocytes, eosinophilic granulocytes and thymocytes but not platelets, erythrocytes and neutrophilic granulocytes.

Staining of leukemic cells and hematopoietic cell lines by mAb K15

mAb K15 stained the leukemic cells of diverse lineages (Table 2). Of the reactive leukemia cell samples, over 90% of the cells stained. The exceptions were two cases of B cell CLL. They were non-reactive to mAb K15 on repeated

determinations. Similarly, mAb K15 stained cells from cell lines of monocytic, myelocytic, erythroid and lymphoid lineages. Of the cell lines tested, more than 75% of cells expressed the K15 antigen except for two B lymphoblastoid cell lines, 8866P and SeD. 51% of 8866P cells stained and 36% of SeD were reactive.

Reactivity of bone marrow cells to mAb K15

The reactivity of bone marrow cells to mAb K15 was analyzed by two methods. In the first method, bone marrow cells were separated into $K15^+$ and $K15^-$ fractions by an immune rosette method. The attached red cells were lysed. $K15^+$ and $K15^-$ cells were fixed and stained with Wright-Geimsa stain. The $K15^+$ cells were lymphocytes, monocytes, myeloblasts, promyeloblasts and esosinophilic granulocytes and the $K15^-$ cells were neutrophilic myelocytes, metamyelocytes and granulocytes, erythroblasts and megakaryocytes (Table 3). Thus, it appears that K15 antigen is expressed by immature cells of the myelocytic series although more mature neutrophilic granulocytes are non-reactive with mAb K15.

The staining of bone marrow cells by mAb K15 was analyzed by flow fluorocytometry. The bone marrow cells were resolved into two populations by light scattering analysis with forward angle and 90° light scattering. One population with more light scattering was arbitrarily termed the "large cell" population while that with less light scattering, the "small cells". These two populations were analyzed with a panel of mAb. It is apparent that the small cell population contained lymphocytes of both T and B cell lineages and most of the $HLA\text{-}DR^+$ cells (Table 4). After the depletion of both T and B cells, variable portions of the small cells stained with mAb K15, mAb Josh 524 (anti-HLA-DR) and Leu 10 (anti-HLA-DC). A small percentage of the large cells were shown to be positive for HLA-DR and the K15 antigen. Whether these cells are the reactive myeloblasts remains to be determined. The staining patterns of the two cell populations by mAb K15 is shown in Figure 3. It is of interest to note that mAb K15 stained a subpopulation of small bone marrow cells brightly. Analysis by log integrated green fluorescence indicated that this was a distinct population.

Antigen K15 is on progenitor cells of CFU-GM and BFU-E

Bone marrow cells were separated into $K15^+$ and $K15^-$ cells by an immune rosette method. The isolated populations were cultured in methylcellulose

supplemented with PHA stimulated PBMC conditioned medium and erythropoietin. CFU-GM and BFU-E were counted after 14 days. As shown in Table 5, the vast majority of the progenitor cells of CFU-GM and BFU-E were in the $K15^+$ fraction. This has been demonstrated in four separate experiments. In two experiments with an agar culture system for the determinations of CFU-GM (7 days), the CFU-GM progenitor cells were also found to be in the $K15^+$ cell fraction. It is also evident that some degree of enrichment for CFU-GM and BFU-E progenitor cells was achieved when cells were separated by this method within Ab K15. For comparison, mAb Josh 524 (anti-HLA-DR) was used to isolate $HLA\text{-}DR^+$ and $HLA\text{-}DR^-$ cell populations. The enrichment of CFU-GM and BFU-E was comparable when the results with mAb K15 and mAb Josh 524 were compared.
Because mAb K15 is a complement fixing antibody, the expression of K15 antigen on CFU-GM and BFU-E progenitor cells was also studied by elimination of $K15^+$ by mAb K15 in the presence of complement. In three experiments, treatment of bone marrow cells by mAb K15 and complement reduced 90-96% of CFU-GM and BFU-E. Thus, these data were in agreement with those presented in Table 5.

Further enrichment of BFU-E progenitor cells

Further enrichment of BFU-E progenitor cells were attempted with mAb K15 and additional monoclonal antibodies. As shown in Table 6, untreated bone marrow cells yielded 242 $\pm$ 12 BFU-E/10^5 cells plated. With the depletion of T and B cells, E^-sIg^- bone marrow cells were enriched for BFU-E progenitor cells. In order to eliminate the monomyelocytic cells and their progenitor cells, mAb H8 which stained monocytes, granulocytes, immature cells in this lineage and CFU-GM progenitors was used. After mAb H8 plus complement treatment, the cells were positively selected for K15 antigen expression. In the resulting $E^-sIg^-H8^-K15^+$ bone marrow cells, ~24-fold enrichment of BFU-E progenitors was observed. A further step was carried out to select $HLA\text{-}DR^+$ cells from the $E^-sIg^-H8^-K15^+$ population, th_ final population was $E^-sIg^-H8^-K15^-HLA\text{-}DR^+$. Approximately 2% of the starting cells were recovered. In this population, 11,500 BFU-E/10^5 plated cells were observed. This represents approximately a 48-fold enrichment. In this experiment, irradiated autologous bone marrow cells were added to provide optimal burst promoting activity.

DISCUSSION

mAb K15 identifies a 22 kD surface antigen on monocytes, lymphocytes of both B and T lineages, immature hematopoietic cells and progenitor cells of CFU-GM and BFU-E. This 22 kD antigen has also been shown to be on AML, AMOL, CML, ALL and CLL cells. It is of interest to note that two of B cell CLL cases were non-reactive, suggesting that a minor B cell population may lack this antigen. Although the progenitor cells of CFU-GM and BFU-E are reactive to mAb K15, erythroblasts, erythrocytes and neutrophilic granulocytes are nonreactive. In addition, eosinophilic granulocytes retain the expression of K15 antigen. The selective loss of a surface antigen during differentiation by cells of certain lineages is of considerable interest. This may provide clues to the function of K15 antigen.

The molecular weight of K15 antigen and the staining pattern of hematopoietic cells by mAb K15 suggest that our antibody identifies a similar antigen as that by mAb S5.7 reported by Pessano, et al. (21). The antigen identified by mAb S5.7 was identified to be a 20 kD protein. It was also reported that 10% of U937 cells were reactive. These minor differences between mAb K15 and S5.7 is probably not real. The minor difference in molecular weight of the reative antigen may be due to the different gel electrophoresis systems and the less reactivity of U937 may be due to different sublines of U937. Definitive identity between these two antigens can be obtained in sequential precipitative experiments involving mAb K15 and S5.7.

The antigen K15 was shown on the progenitor cells of CFU-GM and BFU-E by two different methods. Treatment of bone marrow cells with mAb K15 and complement abolished both CFU-GM and BFU-E. More importantly, positively selected $K15^+$ bone marrow cells were enriched for both CFU-GM and BFU-E progenitor cells. The enrichment was comparable to that of $HLA\text{-}DR^+$ bone marrow cells. Although CFU-GEMM was not determined quantitatively in the present investigation, mixed colonies were observed on several occasions in the $K15^+$ bone marrow cell cultures. Although more definitive experiments are needed, these observations would suggest that K15 antigen may be expressed in more immature hematopoietic cells. Recently, mAb My-10 has been used to enrich for colony forming cells (22). These colony forming cells of different lineages as well as mixed lineages were identified to be larger than

lymphocytes and neutrophiles, round and evenly refractile. Isolation of these cells w d also help to determine if K15 antigen is present on more immature progenitors.

With a combination of monoclonal antibodies against T and B cells, cells of the monocyte-granulocyte lineage and HLA-DR antigen in addition to mAb K15, a bone marrow cell population with 11.5% cloning efficiency for BFU-E progenitor cells was obtained. This degree of enrichment is comparable to the approaches utilized by Lynch and Nathan (7), Civin, et al. (4) and Bodger, et al. (5). Our selection method did not take into consideration K15 antigen density on the bone marrow. As shown in Figure 3, the bone marrow cells could be resolved into K15 bright and K15 dim populations. It would be of interest to determine if future enrichment of BFU-E1 progenitor cells is possible when $K15^+$ cells are sorted into K15 dim and K15 bright populations.

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Table B-1. Expression of K15 surface antigen by peripheral blood cells and thymocytes detected by flow cytometry with mAb K15

Cell types	% staining
Peripheral blood (6)*	
Lymphocytes	95.4 ± 3.3
Monocytes	>80
Granulocytes	4.7 ± 2.5**
Platelets	<1
Erythrocytes	<1
Thymocytes (3)	43.6 ± 4.3

*The number of samples analyzed. Data were presented as the mean of the samples studied.

**The staining cells were shown to be predominantly eosinophiles as shown in Table 2.

Table B-2. Immunofluorescence staining of leukemic cells and cell lines of various hematopoietic lineages by mAb K15

Leukemic Cells	
Acute myeloblastic (6)	++*
Acute monomyeloblastic (1)	++
Chronic myelogenous (2)	++
Non-T acute lymphoblastic (3)	++
T cell acute lymphoblastic (1)	++
B cell chronic lymphocytic (4)	++
(2)	-
T cell chronic lymphocytic (1)	++
Cell lines	
Non-lymphoid lines: ML-1, KG-1, HL-60, HEL, K562 and U937	++
Lymphoblastoid lines: 8402, Molt 4, Jurkat, CEM and MT-1	++
B lymphoblastoid lines: Raji, Daudi, 32a, and Josh-7	++
B lymphoblastoid lines: 8866P and SeD	+**

*++indicates more than 75% of the cells stained. All cell lines were studied on three separate occasions.

**51% of 8866P cells stained and 36% of SeD cells were positive.

Table B-3. Expression of K15 antigen by normal bone marrow cells as detected by mAb K15*

Myeloblasts	+
Promyeloblasts	+
Myelocytes, neutrophilic	-
Metamyelocytes, neutrophilic	-
Neutrophilic granulocytes	-
Eosinophilic granulocytes	+
Erythroblasts	-
Megakaryocytes	-
Lymphocytes	+
Monocytes	+

*Data was obtained by morphological examination of $K15^+$ and $K15^-$ bone marrow cells separated by an immune rosette method.

Table B-4. Expression of the K15 antigen by two populations of bone marrow (BM) cells by light scattering analysis*

Monoclonal antibody	Unfractionated BM cells		E^-sIg^- BM cells	
	Large	Small	Large	Small
K15	4.3**	56.8	5.1	54.4
TE (T11)	<1	48.0	<1	3.5
Josh 524 (HLA-DR)	10.0	27.0	6.1	48.2
Leu 10 (HLA-DC)	<1	17.6	<1	22.3
M3 (anti-μ)	<1	8.5	<1	<1
B1	<1	9.5	<1	<1

*Cell populations were gated on forward angle vs 90° light scattering. The cell population with more light scattering is termed a "large" cell population and that with less light scattering, the "small" cell population. $E^-sIg^-BM^-$ cells were obtained by the depletion of $T11^+$ and μ^+ cells.

**Percent of positive cells. This is a representative experiment of analysis on three bone marrow aspirates.

Table B-5. K15 antigen is expressed on progenitor cells of CFU-GM and BFU-E*

Experiments	Cell population	Colonies/105 cells plated	
		CFU-GM	BFU-E
1	Untreated	77 ± 15	275 ± 16
	$K15^+$	97 ± 4	470 ± 15
	$K15^-$	0	0
	$HLA\text{-}DR^+$	120 ± 7	522 ± 52
	$HLA\text{-}DR^-$		
2	Untreated	101 ± 9	626 ± 31
	$K15^+$	358 ± 33	1138 ± 77
	$K15^-$	2 ± 1	12 ± 4
3	Untreated	96 ± 10	459 ± 27
	$K15^+$	311 ± 85	1425 ± 88
	$K15^-$	15 ± 12	10 ± 14
	$HLA\text{-}DR^+$	461 ± 127	1493 ± 98
	$HLA\text{-}DR^-$	5 ± 4	9 ± 8
4	Untreated	77 ± 12	380 ± 23
	$K15^+$	325 ± 25	1339 ± 104
	$K15^-$	0	1
	$HLA\text{-}DR^+$	191 ± 39	1396 ± 246
	HLA-DR-	1	3

*BM cells were separated into two populations with either mAb K15 or mAb Josh 524 (anti-HLA-DR) by an immune rosette method. Data were presented as the average of four cultures.

Table B-6. Enrichment of progenitor cells of BFU-E with monoclonal antibodies*

Cell population	BFU-E/10^5 cells plated
Untreated	242 ± 12
E^-sIgM^-	439 ± 60
$E^-sIgM^-H8^-K15^+$	5750 ± 540
$E^-sIgM^-H8^-K15^+$-HLA-DR^+	11500 ± 1100

*In this experiment, 10^5 irradiated (1500 rad) autologous bone marrow cells were added to each plate as a source of burst promoting activities. Isolated bone marrow cells were plated at 10^5, 10^4 and 10^3 per plate for BFU-E assays. For isolation of cell populations, mAb TE (T11, receptor for sheep RBC), mAb M3 (anti-μ) were used to obtain E^-sIgM^-BM cells. mAb H8 which stains cells in the GM series and the progenitor cells of CFU-GM was used to obtain $E^-sIgM^-H8^-$ population. $K15^+$ cells were selected positively to obtain the $E^-sIgM^-H8^-K15^+$ population. This population was further selected for HLA-DR^+ cells with mAb Josh 524 to obtain $E^-sIgM^-H8^-K15^+$HLA-DR^+ cells.

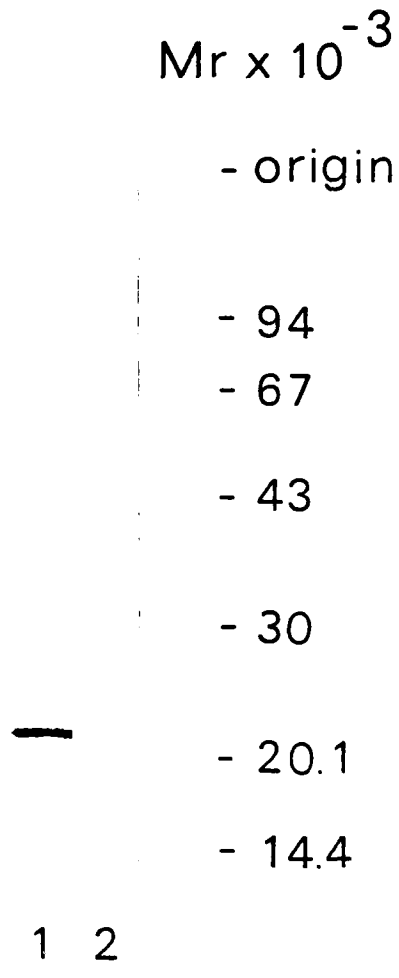

Figure B-1. A 22 kD polypeptide was precipitated by mAb K15 from ^{125}I-labeled K562 cells (Lane 1). No protein was precipitated by a control IgG_3 antibody (Lane 2). 15% polyacrylamide gel was used.

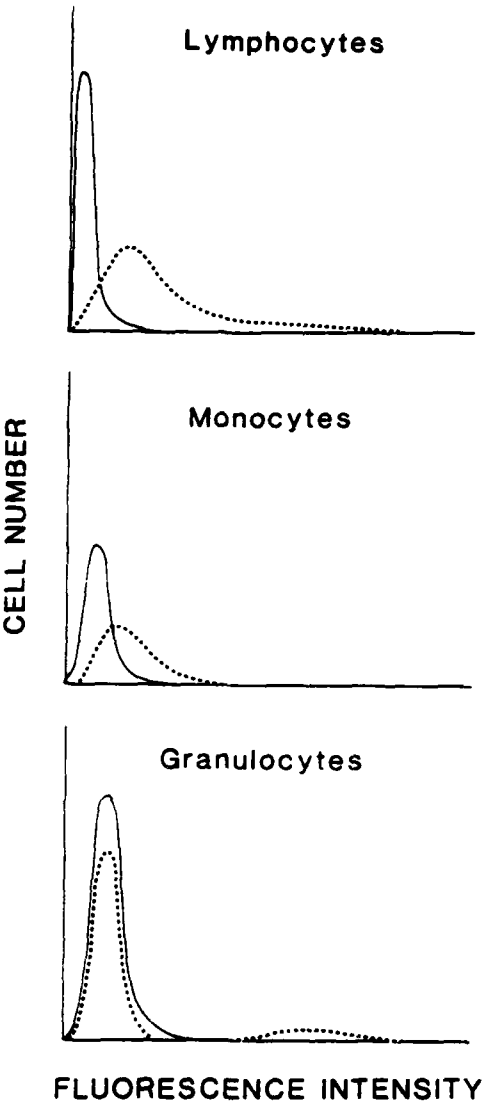

Figure B-2. Fluorocytometric analysis of staining patterns of peripheral blood nucleated cells by mAb K15. Integrated fluorescence was measured by an Epics V instrument with 10,000 cells counted. ___, control mAb; ____ mAb K15.

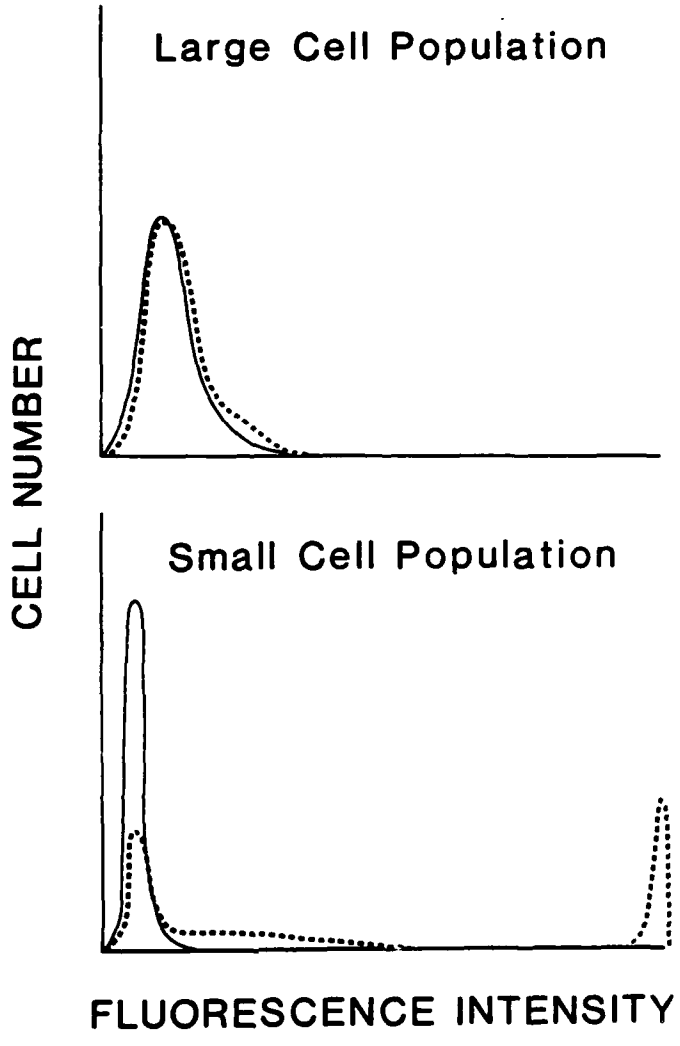

Figure B-3. Staining patterns of E^+sIg^- bone marrow cells by mAb K15. E^-sIg^- bone marrow cells were prepared by the depletion of $T11^-$ (detected by mAb TE) and μ^+ (detected by mAb M3) cells. Fractionated bone marrow cells were gated into two populations by fowrd angle vs 90° light scattering. The cells with more light scattering characteristics was arbitrarily termed "large" cells. The other cell populations was called "small" cells. ____, control mAb; ____ mAb K15.

APPENDEX C

EXPRESSION OF PLATELET ASSOCIATED ANTIGENS BY HEMATOPOIETIC CELLS AND THEIR PROGENITORS[1]

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Running Title: Platelet Associated Antigens

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Abbreviations:
CFU-GM = colony-forming units (granulocyte/monocyte); BFU-E = burst-forming units (Erythroid); PBS = phosphate buffered saline; FBS = Fetal bovine serum mAb = monoclonal antibody; sIg = surface immunoglobulin.

ABSTRACT

A panel of monoclonal antibodies (mAb) were established with HEL, a human erythroleukemia cell line as immunogen. Four of them were shown to be reactive with platelets and megakaryocytes. mAb H229 (IgG_1) precipitated a 43kD glycoprotein from HEL cells and platelets. The reactive antigen appeared to be restricted to platelets and megakaryocytes. mAb H124 (IgM) was reactive with a complex of 140-150kD/90-94kD on HEL cells. In contrast, a single band of 130kD was precipitated from ^{125}I-iodinated platelets. mAb H124 stained mature and nucleated erythrocytes, erythroblasts and a subpopulation of resting and activated T cells. mAb H4 (IgG_{2b}) stained monocytes and precipitated a 140/100kD complex. Ag identified by mAb H5(IgG_{2b}) had a broad cellular distribution. The reactive cells included monocytes, eosinophils, thymocytes, subpopulations of resting and activated T and B lymphocytes and certain myeloblasts. These mAb did not react with significant numbers of neutrophilic granulocytes and their precursors. A small number of bone marrow cells with less light scattering characteristics than myeloid cells, were reactive with mAb H124 and H5. By complement mediated cytolysis and an immune rosette method, antigen H124 was shown to be on BFU-E and H5 on CFU-GM. Studies with leukemia cells and cell lines yielded results, generally reflective of antigen distribution on normal cells. However, cells of two CML patients who were not in blastic crisis, stained with three of these mAb (H229, H124 and H5). The relationship between these mAb and those previously reported and the utilization of these mAb and HEL to probe hematopoiesis are discussed.

INTRODUCTION

Monoclonal antibodies (mAb) specific for the major human platelet glycoprotein complexes (Ia/Ib and IIb/IIIa) have been reported and utilized to probe the function of these complexes (1-12). In the case of the IIb/IIIa glycoprotein complex, it has been assumed to be preferentially expressed by platelets. Recent evidence indicates that this complex may also be synthesized by monocytes (13). Monoclonal antibodies against other shared determinants between platelets and various hematopoietic cells have also been established. These include antigens common on platelets and monocytes (13,14), on platelets and common acute lymphoblastic leukemia (ALL) cells (15,16) and on platelets, monocytes and nucleated erythrocytes (17).

Recently, attempts to generate monoclonal antibodies to non-lymphoid cell lines in our laboratory have lead to the establishment of monoclonal antibodies reactive with CFU-GM and BFU-E progenitors (18,19). With HEL, a human erytholeukemia cell line (20) as immunogen, several monoclonal antibodies reactive with platelets were encountered. Four of them (mAb H229, mAb H124, mAb H4 and mAb H5) have been characterized. They were reactive with megakaryocytes. In addition, their reactivities to hematopoietic cells and certain progenitors (CFU-GM and CFU-E) presented a distinct pattern of platelet associated antigen expression.

MATERIAL AND METHODS

Monoclonal Antibodies

HEL, a human erythroleukemia cell line was kindly provided by Dr. P. Martin of the Fred Hutchinson Cancer Research Center, Seattle, WA. Ten week old BC_3F_1 or Balb/C mice were injected with 2 x 10^7 HEL cells intraperitoneally (i.p.) with 4 mg alum as adjuvant. Three weeks later, 2 x 10^7 HEL cells were injected i.p. in phosphate buffer saline (PBS). Spleens were taken three days later for fusions with SP2/0 Ag14 tumor cell line with PEG1000. Their supernatants were screened by indirect immunofluorescence. The desired hybridomas were cloned on agarose with fibroblasts as feeders. Details of these procedures have been described previously (21).

Other monoclonal antibodies used in the present studies are mAb Josh 524, mAb M3, mAb TE, mAb H8, Leu10 and B1. The first four antibodies were generated in our laboratory. mAb Josh 524 (IgG_1) is specific for HLA-DR. It precipitated a 29/34 kD bimolecular complex. mAb M3 (IgG_1) is specific for μ chain. mAb TE (IgG_2) blocked rosette formation between T cells and sheep erythrocytes. It precipitated a 55kD glycoprotein. It is equivalenc to anti-T11 mAb. Leu10, an anti-HLA-DC antibody, was obtained from Becton-Dickinson, Mountain View, CA. B1, a B cell specific mAb, was obtained from Coulter Immunology (Hialeah, FL). As appropriate control mAb, NS8-1 (IgG_{2b} , anti-sheep RBC), NS4.1 (IgM, anti-sheep RBC) and HDP-1 (IgG_1, anti-DNP) are used. These antibodies were kindly provided to us by Dr. J. Davie of Washington University, St. Louis, MO.

Cell Preparation

Defibrinated blood from normal volunteers was used as a source of peripheral blood mononuclear cells (PMNC). PMNC were isolated by Ficoll/Hypaque gradient centrifugation. T cells and non-T cells were separated by a rosette formation method with sheep red cells. Phagocytic cells were removed from non-T cell preparation by the incubation of non-T cells with carbonyl iron and the removal of iron-ingested cells with a magnet. These procedures were described previously (22). Monocytes were separated by Percoll continuous gradient centrifugation (23). To minimize the attachment of platelets to monocytes, all solutions contained 5mM EDTA. The monocytes were washed with PBS, 5mM EDTA prior to being used for indirect

immunofluorescence. Granulocytes were isolated from the peripheral blood as the pellet fraction after Ficoll-Hypaque density gradient centrifugation. Contaminating erythrocytes were removed by centrifugation on a 60%, 65% and 70% Percoll discontinuous density gradient. The purify of the granulocyte fraction was more than 94% as determined by Wright-Giemsa staining. Platelets were isolated from citrated platelet-rich plasma and erythrocytes from the pellets of Ficoll-Hypaque gradients of peripheral blood cells.

Leukemia cells of different lineages were isolated from the peripheral blood of the affected patients by Ficoll-Hypaque gradient centrifugation. They were cryopreserved and recovered prior to staining.

Cell Lines

In addition to HEL, U937, a histocytic/monocytoid cell line; ML-1 and KG1, myeloblastic cell lines; K562, an erythroid/myeloid line from a patient with CML in blastic crisis; and HL-60, a promyelocytic cell line, were used as nonlymphoid lines. For T leukemia cell lines, RPMI-8402, MT-1, Molt-4, Jurkat and CEM were used. As B lymphoblastoid cell lines, Raji, Daudi, Josh-7, a pre-B cell line (24), 8866P (IgG); 32a1, (IgA producing) and SeD (25) were used. All cell lines were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS).

Bone Marrow Cells and Bone Marrow Cultures

Bone marrow cells were obtained from normal volunteers. They were separated by Ficoll-Hypaque density centrifugation to remove erythrocytes and mature granulocytes. Separated bone marrow cells were subjected to an immune rosette method (26) to obtain cells reactive with the monoclonal antibody of interest or they were subjected to a complement mediated cytolysis procedure to deplete the reactive cells. In the immune rosette method, 2×10^7 bone marrow cells were incubated with the supernatant containing the mAb under investigation for 30 minutes at 4°C. After three washings with RPMI- 1640 and 10% FBS, the bone marrow cells were incubated with 10 human AB red cells coated with $F(ab')_2$ fragments of affinity purified goat antibodies specific for mouse Ig for 30 minutes at 4°C. The rosetting cells were separated from the non-rosetting cells by Ficoll-Hypaque centrifugation. The red cells in the pellet were lysed with buffered $(NH_4)_2SO_4$. For the complement mediated cytolysis procedure, 0.5 ml of 2×10^7/ml of bone marrow cells were incubated

with 0.5 ml of hybridoma culture supernatant for 30 min at 37°C. 1.0 ml of baby rabbit complement (Pel Freez Bilogicals, Rogers, AR) was added and the mixture was incubated for 45 min at room temperature. Dead cells were removed by centrifugation on a Ficoll-Hypaque gradient. Bone marrow cells were cultured in quadruplicates in Iscove's modified Dulbecco's medium containing 0.9% methyl-cellulose, 10% conditioned medium from human peripheral blood leukocytes stimulated with phytohemagglutinin (PHA-LCM) and 30% FBS. One unit of erythropoietin (sheep fraction III, Connaught, Toronto, ONT) was added to each plate. This procedure was essentially that described by Messner, et al. (27). Hemoglobin-containing single or multiple colonies of greater than 64 cells on the 14th day were scored as BFU-E. This culture system also allowed CFU-GM colony formation. Colonies of greater than 40 cells on the 14th day were scored as CFU-GM.

Immunofluorescence Studies

Cells ($0.05-1.0 \times 10^6$) were first incubated with hybridoma culture supernatants for 20 min. After three washings with 0.01 M phosphate buffered saline with 1% bovine plasma albumin, fluorescein labeled $F(ab')_2$ of affinity purified goat anti-mouse Ig antibodies were added and a 20 min incubation at 4°C was carried out. After three washing, cells were analyzed with a Coulter Epics V flow cytometer (Coulter Electronics, Hialeah, FL). Integrated fluorescence of the population gated by forward angle light scatter and right angle light scatter was measured and 10,000 cells were analyzed.

Iodination and Immunoprecipitation

Cells were iodinated in suspension by the method of Hubbard and Cohn (28). Briefly, 1×10^{10} platelets, 2×10^7 HEL cells or 2×10^7 monocytes were incubated with 1 mCi/ml Na ^{125}I, 50 mU/ml type V glucose oxidase (Sigma, St. Louis, MO), and 10 µg/ml lactoperoxidase (LPO; Calbiochem-Behring Corp., San Diego, CA) for 15 min on ice. The reaction was terminated by aspirating the supernatant and repeated washings with RPMI-1640. After iodination, immunoprecipitation, gel electrolysis and autoradiography were performed as described previously (29).

RESULTS

Characterization of mAb H229, H124, H4 and H5

Hybridoma H229, HI24, H4 and H5 were generated from two independant fusions. Hybridoma H4 was a fusion product between a BC_3F_1 spleen cell and a SP2/0 Ag14 cell. The other three hybridomas were from a fusion with Balb/C spleen cells. By ELISA, mAb H229 was typed to be IgG_1; mAb H124, IgM; mAb H4 and H4, IgG_{2b}. Except for mAb H229, these mAb fixed complement.

With ^{125}I labeled HEL cells and under reduced condition for SDS gel electrophoresis, mAb H229 precipitated a 43kD glycoprotein (Figure 1, Lane 1). A similar glycoprotein was precipitated from platelets. mAb 124 brought down two broad bands in the regions of 140-150kD and 90-94kD (Figure 1, Lane 2). These two broad bands were also seen when the gel electrophoresis was run under non-reduced condition. In contrast to HEL, a single band of 130kD was precipitated by mAb H124 from ^{125}I labeled platelets (Figure 1, Lane 3). This difference in antigenic structures on HEL and platelets was demonstrated in three other experiments. mAb H4 precipitated a non-covalently linked dimer of 140kD/100kD (Figure 1, Lane 5). The upper band was much less well labeled. This observation was seen in two other immunoprecipitation experiments. In one experiment, only the 140kD component was precipitated. In the case of mAb H4, a 26kD polypeptide was identified (Figure 1, Lane 7). For mAb H4 and H5, similar glycoproteins were precipitated from ^{125}I labeled monocytes and platelets.

Reactivity with Peripheral Blood Cells and Thymocytes

The cellular distribution of the reactive antigens by mAb H229, H124, H4 and H5 was analyzed by indirect immunofluorescence with an Epic V flow cytometer. From peripheral blood, platelets, monocytes, granulocytes, lymphocytes and erythrocytes were isolated. These cell preparations contained greater than 90% of the desired cells. The cells were first gated by forward angle and 90° light scattering. Integrated green fluorescence was measured on the gated population. The results are summarized in Table 1 and shown in Figures 2 and 3. All four antibodies stained platelets well. mAb H4 gave the most intense staining with most if not all the platelets at the last channel of fluorescence intensity. The other three mAb showed heterogeneious staining intensities within the platelet population (Figure 2).

For peripheral blood nucleated cells, both mAb H224 and H4 did not stain a significant number of lymphocytes. mAb H5 stained a small population (7.6 ± 3.6) weakly whereas mAb H124 stained a small population ($6.8\pm5.9\%$) brightly (Figure 3A). The reactive lymphocyte populations with mAb H5 and H124 varied greatly in numbers for different individuals. Although these monoclonal antibodies were of different isotypes, control antibodies of different isotypes gave similar staining patterns. For simplicity, only one control curve is included in each panel of Figure 3. In the case of monocytes, no significant staining was seen with mAb H229 and H124. Both mAb H4 and H5 gave considerable staining on the majority of the monocytes (Figure 3B). For granulocytes, three mAb (H229, H124 and H4) showed no reactivity. mAb H5 stained $7.5\pm3.6\%$ of the granulocytes in six determinations. The staining of this minor population was distinct and intense (Figure 3C). The granulocytes which were incubated with mAb H5 and formed rosettes with human red cells coated with $F(ab')_2$ of goat anti-mouse Ig, were identified to be eosinophils by Wright-Geimsa staining.

The reactivity of these mAb with thymocytes were also studied. mAb H5 stained $29.0\pm9.6\%$ of the thymocytes in three separate experiments. The other three mAb did not react significantly with these cells. mAb TE (T_{11}) and Josh 524(HLA-DR) were included in Table 1 to show some of the characteristics of the cell populations.

Expression of Platelet Associated Antigens on Activated T and B Cells

As shown in Table 2, mAb H124 stained a small population of T cells but not B cells in a representative experiment. In contrast, mAb H5 reacted with a subpopulation of T and B cells. Both mAb H229 and H4 failed to stain isolated T and B cells. This agrees with their nonreactivity with lymphoid cells. Both mAb H124 and H5 were reactive with substantial numbers of Con A activated T cells. mAb H5 also stained a portion of activated B cells which were treated with anti-μ and conditioned medium containing B cell growth factor (30). Although not presented, two additional experiments gave similar results.

Staining Patterns on Hematopoietic Cell Lines and Leukemic Cells

Antigen expression by various human hematopoietic cell lines is shown in Table 3. Of six non-lymphoid lines, four mAb stained HEL cells, the immunogen.

mAb H229, H124 and H4 stained greater than 95% HEL cells. However, only 41.7±4.7% of HEL cells were stained by mAb H5. mAb H229 stained KG-1 cells. mAb H124 reacted with a population of K562 and KG-1 cells. mAb H4 and H5 did not stain the other five non-lymphoid cell lines. Of five T leukemia cell lines, mAb H224 stained a small population of Jurkat and CEM cells, whereas mAb H5 stained a major population of 8402, MT-1 and Molt-4 cells. The other two mAb did not stain any of these T cell line cells. All four mAb failed to react with five B cell lines with one exception. mAb H229 stained the majority of the cells in an IgA producing cell line 32a1.

Leukemic cells of both myeloid and lymphoid origin were examined for the expression of these platelet associated antigens (Table 4). mAb H229, H124 and H4 stained a minor population of certain AML blasts whereas mAb H5 stained varying numbers of AML blasts in all six cases. In a case of acute myelomonocytic leukemia, mAb H124 and H5 stained 27.7% and 89.0% of the cells respectively. Cells of two cases of CML were reactive with mAb H229, H124 and H5 but not with H4. mAb H4 and H5 were reactive with blasts of three cases of ALL, one of which was of T cell origin. Despite its non-reactivity with B lymphoblastoid cell lines, mAb H5 was reactive with varying numbers of leukemic B cells in six of seven CLL cases. The other three antibodies did not react with any CLL cells.

Antigenic Expression on Bone Marrow Cells.

The reactivity of bone marrow cells were studied. Bone marrow cells were reactive with mAb under investigation and the reactive cells were isolated with human erythrocytes coated with $F(ab')_2$ of goat anti-mouse Ig antibodies by an immune rosette method. The separated reactive and non-reactive cells were stained with Wright-Geimsa stain and examined morphologically. Aa shown in Table 5, all four mAb were reactive with megakaryocytes. Except for mAb H5, none of these mAb was reactive with cells in the myeloid lineage. mAb H5 reacted with myeloblasts and eosinophiles and their precursors. Of the four mAb, only mAb H124 reacted with both mature and inmature erythroid cells. Both mAb H4 and H5 reacted with monocytes. A significant number of the lymphocytes were also reactive with mAb H124 and mAb H5.

The staining of bone marrow cells was also analyzed by flow cytometry. The bone marrow cells were resolved into two populations by forward angle and 90° light scattering analysis. One population with more light scattering was arbitrarily termed the "large cell" population while that with less light

scattering, the "small cell" population. These two populations were analyzed with a panel of mAb (Table 6). In the case of unseparated bone marrow samples which were often contaminated with peripheral blood, the ratio between cell numbers of the "large cell" and "small cell" populations was approximately 1:1. The small cell population contained lymphocytes of B and T cell lineages and most of the HLA-DR$^+$ cells. The big cell population contained predominantly cells of the myelomonocytic lineage. This was shown with a panel of mAb specific for myloid and monocytic cells (19). With depletion of T and B cells, the ratio between the big and small cells was approximately 4:1. As shown in Table 6, none of the mAb H229, H124, H4 and H5 stained substantial numbers of large cells. mAb H229 did not stain cells in the small cell population while the other three mAb stained a minor population of small cells. Significantly, mAb H124 and H5 identified a subpopulation of small cells with bright staining intensities (Figure 4). It remains to be determined if these reactive populations contain cells with progenitor potential.

Presence of Antigen H124 on BFU-E and Antigen H5 on CFU-GM Progenitors

mAb H124, H4 and H5 fixed complement. Using complement mediated cytolysis, antigen H124 was found to be present on BFU-E (Table 7). In one experiment, over 95% BFU-E reduction was observed when the bone marrow cells were treated with mAb H124 plus complement. In another, only 60% reduction was seen. Similarly, 90-80% CFU-GM reduction was seen when bone marrow cells were treated with mAb H5 and complement. mAb H4 had no effect on CFU-GM or BFU-E in this assay system.

These findings were confirmed by an alternative cell seperation method. As shown in Table 8, H124$^+$ cells isolated by an immune rosette method contained the vast majority of the BFU-E progenitors. In the experiment in which a significant number of BFU-E was found in the H124$^-$ cell population, the burst size was much larger. This may indicate tnat more immature BFU-E progenitors express less antigen. It is also of interest that certain enrichment for BFU-E was achieved by this method. The degree of enrichment was similar to that with our anti-HLA-DR mAb. In the case of mAb H5, CFU-GM was found predominantly in the H5$^+$ population, while BFU-E in the H5$^-$ population. Both CFU-GM and BFU-E were found to be in the H229$^-$ or H4$^-$ population, indicating the absence of these antigens on CFU-GM and BFU-E progenitors. As a control, all CFU-GM and BFU-E were found in the HLA-DR$^+$ population.

DISCUSSION

Four monoclonal antibodies specific for platelet associated antigens have been established with HEL cells as immunogen. They are reactive with megakaryocytes as well as platelets. Cellular distribution and immunoprecipitation studies show that they are reactive with distinct antigenic determinants. Except for the possible relationship between the H4 antigen and the platelet glycoprotein complex, IIb/IIIa, these antigens appear not to be associated with the major platelet glycoprotein complexes. These mAb will be useful for the further investigation of hematopoiesis.

mAb H229 identifies a 43kD glycoprotein from both HEL cells and platelets. Although the expression of H229 was demonstrated in certain cell lines and CML cells, its expression in normal hematopoietic cells was limited to platelets and megakaryocytes. To our knowledge, this molecule has not been described. Thus, mAb H229 identifies a novel platelet specific antigen.

The distribution of antigen H124 is limited to platelets, megakaryocytes, erythrocytes and a subset of resting and activated T cells. Its broader reactivities toward leukemic cell lines reflect this broad cellular distribution. mAb H124 precipitated two broad bands of 140-150kD and 90-94kD. In contrast to this precipitated complex from HEL, a single 130kD band was precipitated from platelets. One possible explanation for the observation is that mAb H124 is reactive with a determinant that is primarily determined by a carbohydrate structure on glycoproteins. The polypeptides on HEL and platelets are not related except that glycosylation provides a common determinant. This is endogenous to the antigen 1G10 (31). mAb 1G10 identifies a 180kD glycoprotein on untreated HL-60 cells and a 150kD glycoprotein on differentiated HL-60 cells treated with retinoic acid. The 1G10 antigenic determinant has been identified to be the carbohydrate hapten with the structure, Galβ1-4(Fucα1-3)GlcNAcβ1-R. An alternative explanation is that the band of 130kD from platelets is related to the broad band 140-150kD. The difference in mobilities is due to further modification of the peptide backbone structure during differentiation. Peptide mapping of these two structures will either support or dispute this hypothesis.

mAb H4 reacts with platelets, megakaryocytes and monocytes but not with other bone marrow cells, leukemic cells or various hematopoietic cell lines. Its reactive antigen is a 140/100kD bimolecular complex. The 140kD molecular

moiety has been shown to be consistantly less iodinated in our system. The cellular distribution and the nature of the antigen suggest that antigen H4 is very similar to the MPA antigen reported by Burchhardt, et al. (14). The isolation of this antigen from HEL cells and from monocytes isolated from citrated blood by Percoll gradiant centrifugation rules out the possibility that the antigen is derived from platelets often adhered to monocytes due to in vitro activation. The relationship between H4 antigen and the glycoprotein complex IIb/IIIa is worthy of further exploration because of the similarity of molecular weights between these two complexes. This is particularly relevent in view of the staining of HEL cells by an anti-IIb/IIIa mAb (32). The recent demonstration that certain mAb specific for the IIb/IIIa complex stain monocytes (13) further supports the possibility that H4 antigen is the IIb/IIIa complex. Sequential immunoprecipitation experiments involving mAb H4 and mAb specific for the IIb/IIIa complex will resolve this question.

Of the four monoclonal antibodies reactive with platelets, mAb H5 has the broadest cellular distribution. It reacts with platelets, megakaryocytes, monocytes, eosinophiles and subpopulations of thymocytes, resting and activated T and B cells. It stains most leukemic cells of diverse lineages. mAb H5 precipitates a 26kD molecule from either platelets or HEL cells. The molecular weight of H5 is very similar to those identified by mAb DU-ALL-1 (15), CALL-1 (16), FMC-8 (33), BA-2 (34) and TP82 (35). All these antigens have been identified to be 24kD. Although cellular distribution of H5 differs from those of the above antigens, direct comparison between these mAb is needed to allow definitive conclusions regarding identity between these antigens.

HEL, the immunogen was established from a patient with acute erythroleukemia (20). This cell line has been shown to be capable of spontaneous and induced globin synthesis. It is also inducible to undergo a drastic macrophage-like shift with the phorbol ester, TPA (32). Papayannopoulou and coworkers have also produced a series of monoclonal antibodies against HEL (36). Some of them are reactive with platelets and progenitors of CFU-GM and CFU-E. The generation of anti-platelet antibodies with HEL as an immunogen in the present investigation and the expression of myelomonocytic specific antigens as reported (19) add further support that HEL is derived from a multipotent progenitor cell. Unlike K562, an erythroid/ myeloid cell line with multiple lineage surface markers (37-42), HEL expresses

HLA-DR antigens. It is possible that HEL may also express antigens preferentially expressed by the human multipotent stem cells. Further utilization of HEL as an immunogen to generate mAb specific for these cells is warranted.

The expression of three of the platelet associated glycoproteins by CML cells is an unexpected finding (Table 4). This is particularly surprising because only one of the mAb stained identifiable immature cells in the granulocytic series. Since these two CML patients were not in blastic crisis and their cells expressed myelomonocytic antigens (19), this finding adds further support to the thesis that there is dyshemotopoiesis involving the multipotent stem cells in CML. A previous study with an anti-platelet antibody in the study of CML in blastic crisis shows only a rare patient with blasts expressing both myeloid and platelet markers (43). It is likely that the employment of multiple markers of a single lineage may provide a more accurate estimation of the potentials of these CML blasts.

The expression of these platelet associated antigens by hemotopoietic progenitor cells was investigated on a limited scale. Antigen H124 was expressed on BFU-E but not on CFU-GM. Antigen H5 was on FU-GM but not on BFU-E. Neither H229 nor H4 was on these progenitors. It would be of considerable interest to determine whether these antigens are expressed by progenitors of megakaryocyte colonies according to the method of Messner, et al. (44) and those of the mixed cell (CFU-GEMM) colonies (45).

The expression of H5 by eosinophiles requires further comments. Although this antigen is not expressed by neutrophilic granulocytes and their precursor, it is expressed on eosinophiles in a readily detectable quantity. A 22kD protein which is identified by a mAb, K15 generated in our laboratory has a similar distribution on granulocytes and their precursors (17) mAb K15 stains eosinophilic granulocytes but not neutrophiles although it also reacts with promyeloblasts and myeloblasts. mAb K15 stains the majority of the monocytes. Both K15 and H5 antigens are expressed on CFU-GM. The selective retention of K15 and H5 antigens on monocytes and eosinophiles gives clues to the possible function of these antigens. These findings also provide supportive evidence that CFU-GM and CFU-E are distinct.

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Table C-1. Antigenic expression by peripheral blood cells and thymocytes as detected by anti-HEL mAb.

Cells	Monoclonal Antibody					
	H229	H124	H4	H5	TE(T11)	Josh 124(HLA-DR)
Peripheral Blood (6)*						
Platelets	31.3±2.4(%)**	84.5±5.5	93.1±5.4	94.0±3.8	<1	<1
Monocytes	<1	<1	91.4±1.5	92.6±2.7	3.0±1.8	92.6±2.8
Granulocytes	<1	<1	<1	7.5±3.6	<1	<1
Lymphocytes	<1	6.8±5.9	<1	13.0±3.0	87.4±2.9	11.6±3.3
Erythrocytes	<1	>99+	<1	<1	<1	<1
Thymocytes (3)	<1	<1	<1	29.0±9.6	88.5±8.4	<1

*The number of cell samples were analyzed.

**Data are presented as mean ± standard deviation.

+All erythocytes were stained and most were aggregated when examined by fluorescence microscopy.

Table C-2. Antigenic Expression of H124 and H5 by resting and activated T and non-T cells

Monoclonal Antibody	Cell type			
	T	B	Activated T	Activated B*
H124	7.6(%)	2.0	41.9	<1
H5	13.0	46.1	73.0	42.1
H229	<1	<1	<1	<1
H4	<1	<1	2.7	<1
TE (Sheep RBC Receptor)	98.6	2.6	95.8	10.4
Josh 524 (HLA-DR)	4.8	95.3	N.D.	N.D.
H8 (anti-Myelomonocytic)	<1	<1	<1	<1

*61% of the activated B cells were positive for B1.

Table C-3. Antigenic Expression by various hematopoietic cell lines.

Cell line	Cell type	Monoclonal Antibody			
		H229	H124	H4	H5
HEL	Erythroleukemia	97.6±1.6	98.3±2.1	97.8±1.9	41.7±4.7
K562	Erythroid/Myeloid	<1	56.8±7.3	<1	<1
KG-1	Myeloblastic	91.2±3.7	28.2±5.9	<1	<1
ML-1	Myeloblastic	<1	<1	<1	<1
HL-60	Promyelocytic	<1	<1	<1	<1
U937	Histocylic/Monocytoid	<1	<1	<1	<1
8402	T leukemia	<1	<1	<1	72.1±5.3
MT-1	T leukemia	<1	<1	<1	97.1±1.1
Molt-4	T leukemia	<1	<1	<1	83.0±3.9
Jurkat	T leukemia	<1	34.0±5.9	<1	<1
CEM	T leukemia	<1	14.9±2.6	<1	<1
Raji, Daudi	Burkitt's lymphoma	<1	<1	<1	<1
Josh 7	Precursor B cell	<1	<1	<1	<1
8866P	B lymphoblastic	<1	<1	<1	<1
SeD	CLL	<1	<1	<1	<1
$32a_1$	B lymphoblastic	82.2±6.7	<1	<1	<1

Data are presented as mean ± standard deviation from 3 experiments.

Table C-4. Staining of Leukemias of different lineages by anti-HEL mAb.

	Monoclonal Antibody			
	H229	H124	H4	H5
Acute Myelogenous Leukemia (AML)				
1	<1(%)	<1	<1	89.0
2	<1	12.4	<1	90.1
3	<1	16.4	12.9	45.1
4	<1	<1	<1	21.8
5	10.1	<1	<1	32.9
6	<1	<1	10.0	31.8
Acute Myelomonocytic Leukemia (AMML)	<1	27.7	<1	89.0
Chronic Myelogenous Leukemia (CML)				
1	60.2	15.5	<1	67.2
2	76.5	67.5	<1	42.3
Acute Lymphocytic Leukemia (ALL)				
1 T cell	<1	25.3	<1	53.3
2 non-T, non-B	<1	14.7	<1	86.7
3 non-T, non-B	<1	5.7	<1	53.3
Chronic Lymphocytic Leukemia (B Cell) (CLL)				
1	<1	<1	<1	38.6
2	<1	<1	<1	53.0
3	<1	<1	<1	9.5
4	<1	<1	<1	44.8
5	<1	<1	<1	68.2
6	<1	<1	<1	77.2
7	<1	<1	<1	<1

Table C-5. Expression of H229, H124, H4 and H5 antigens by bone Marrow Cells.

	Monoclonal Antibody			
Cell type	H229	H124	H4	H5
Megakaryocyte	+	+	+	+
Myeloblasts	-	-	-	+
Promyeloblast	-	-	-	-
Myelocyte	-	-	-	-
Metamyelocyte	-	-	-	-
Neutrophile	-	-	-	-
Eosinophile	-	-	-	+
Erythroid - blast	-	+	-	-
- nucleated	-	+	-	-
Monocyte	-	-	+	+

Table C-6. Antigenic expression by two populations of bone marrow cells resolved by light scattering analysis.

	Unfractionated Bone Marrow		E^-sIg^- Bone Marrow Cells	
Monoclonal Antibody	Large	Small	Large	Small
H229	1.9 ± 1.6(%)*	<1.0	<1.0	<1.0
H124	<1.0	13.2 ± 5.5	<1.0	7.5 ± 3.0
H4	5.1 ± 3.6	2.3 ± 2.6	2.9 ± 0.3	6.4 ± 4.3
H5	3.7 ± 0.1	16.2 ± 2.3	<1.0	31.2 ± 2.8
TE (Sheep RBC Receptor)	<1.0	48.0 ± 6.6	<1.0	3.5 ± 2.7
Josh 524 (HLA-DR)	10.1 ± 7.5	27.0 ± 4.5	6.1 ± 5.8	48.2 ± 17.3
Leu 10 (HLA-DC)	<1.0	17.6 ± 4.0	<1.0	22.3 ± 12.8
M3 (anti-μ)	<1.0	8.5 ± 3.5	<1.0	<1.0
B1	<1.0	9.5 ± 4.0	<1.0	<1.0

Data were presented as mean $\pm$ standard deviation of 3 separate experiments.

Table C-7. Expression of H124 on BFU-E progenitors and H5 on CFU-GM progenitors respectively as demonstrated by complement (C') cytolysia.

Experiment	Bone Marrow Cell Treatment	Colonies/10^5 cell plated	
		BFU-E	GFU-GM
1	Untreated	86±16	87±4
	C' alone	98±34	97±8
	C' + mAb124	3±2	105±4
2	Untreated	82±31	97±27
	C' alone	83±9	98±13
	C' + mAb124	31±13	105±8
3	Untreated	245±12	163±7
	C' alone	276±71	172±26
	C' + mAb124	51±10	173±7
	C' + mAb H5	260±34	12±4
4	Untreated	242±12	76±6
	C' alone	224±35	83±5
	C' + mAb H5	229±14	16±7
	C' + mAb H4	321±20	83±16
5	Untreated	88±14	46±7
	C' alone	106±40	52±7
	C' + mAb H4	133±42	60±21

*Data were presented as the mean ± standard deviation of quadruplicate cultures.

Table C-8. Expression of Antigen H229, H124, H4 and H5 by BFU-E and CFU-GM Progenitors.

Bone Marrow all type	BFU-E/10^5 cells plated		CFU-GM/10^5 cells plated	
	I	II	I	II
Unseparated	$275^{\pm}16$*	$358^{\pm}41$	$77^{\pm}15$	$117^{\pm}35$
$H229^+$	$53^{\pm}19$	$36^{\pm}7$	$28^{\pm}9$	$12^{\pm}11$
$H229^-$	$340^{\pm}58$	$452^{\pm}29$	$78^{\pm}19$	$132^{\pm}19$
$HLA\text{-}DR^+$	$522^{\pm}132$	$1079^{\pm}62$	$120^{\pm}7$	$343^{\pm}11$
$HLA\text{-}DR^-$	$1^{\pm}1$	$3^{\pm}1$	$2^{\pm}1$	$3^{\pm}2$
Unseparated	$358^{\pm}41$	$459^{\pm}27$	$117^{\pm}35$	$96^{\pm}10$
$H124^+$	$1077^{\pm}75$	$1720^{\pm}369$	$24^{\pm}8$	$24^{\pm}3$
$H124^-$	$53^{\pm}27$	$1^{\pm}1$	$122^{\pm}50$	$118^{\pm}27$
$HLA\text{-}DR^+$	$1079^{\pm}62$	$1493^{\pm}98$	$343^{\pm}11$	$461^{\pm}127$
$HLA\text{-}DR^-$	$3^{\pm}1$	$9^{\pm}8$	$3^{\pm}2$	$5^{\pm}4$
Unseparated	$242^{\pm}12$	$358^{\pm}41$	$76^{\pm}6$	$117^{\pm}35$
$H4^+$	$28^{\pm}5$	$66^{\pm}10$	$17^{\pm}5$	$17^{\pm}12$
$H4^-$	$327^{\pm}24$	$444^{\pm}71$	$115^{\pm}22$	$153^{\pm}12$
$HLA\text{-}DR^+$	$303^{\pm}24$	$1079^{\pm}62$	$142^{\pm}19$	$343^{\pm}11$
$HLA\text{-}DR^-$	$23^{\pm}4$	$3^{\pm}1$	$2^{\pm}1$	$3^{\pm}2$
Unseparated	$459^{\pm}27$	$380^{\pm}23$	$96^{\pm}10$	$77^{\pm}12$
$H5^+$	$60^{\pm}22$	$28^{\pm}12$	$296^{\pm}67$	$90^{\pm}7$
$H5^-$	$453^{\pm}22$	$383^{\pm}22$	$22^{\pm}17$	$22^{\pm}3$
$HLA\text{-}DR^+$	$1493^{\pm}98$	$1396^{\pm}246$	$461^{\pm}127$	$191^{\pm}39$
$HLA\text{-}DR^-$	$9^{\pm}8$	$3^{\pm}5$	$5^{\pm}4$	$1^{\pm}1$

*Data were presented as the mean $\pm$ standard deviation of quadruplacate cultures.

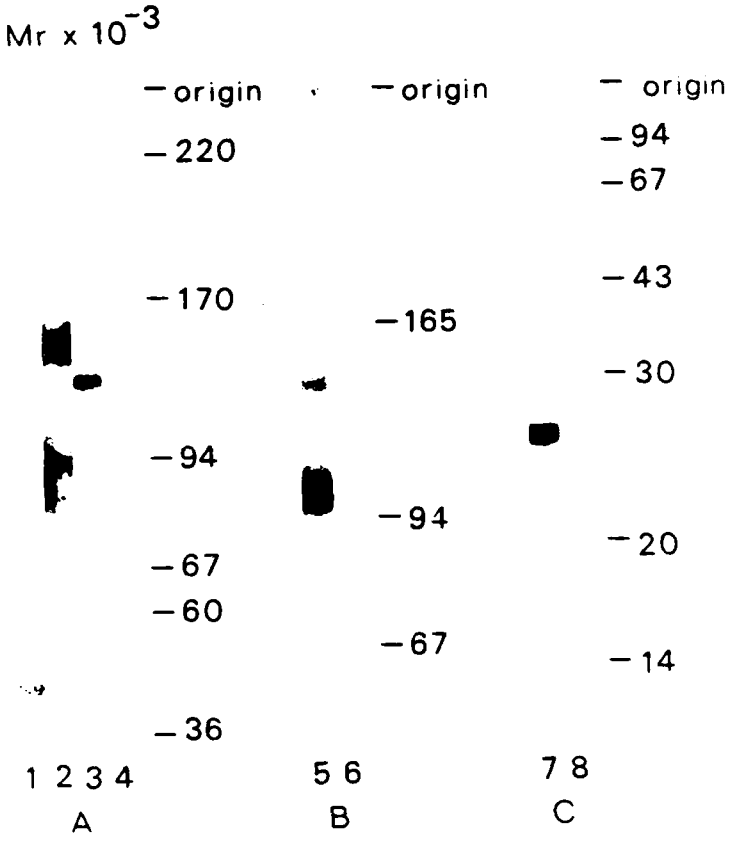

Figure C-1. Reactive antigens from HEL cells and platelets. Panel A. Immunoprecipitates were run on a 9% SDS polyacrylamide slab gel. mAb H229 precipitated a 43kD molecule from HEL (Lane 1). mAb H124 precipitated two broad bands of 140-150kD and 90-94kD from HEL cell lysate (Lane 2) while a 130kD molecule was precipitated from platelets (Lane 3). Panel B. Immunoprecipitate by mAb H4 was run on a 4-11% gradient gel. A 140/100kD bimolecular complex was precipitated from HEL (Lane 5). Panel C. Immunoprecipitate was run on a 15% gel. A 26kD protein was precipitated by mAb H5 HEL (Lane 7). Lanes 4, 6 and 8 were immunoprecipitates with control mAb.

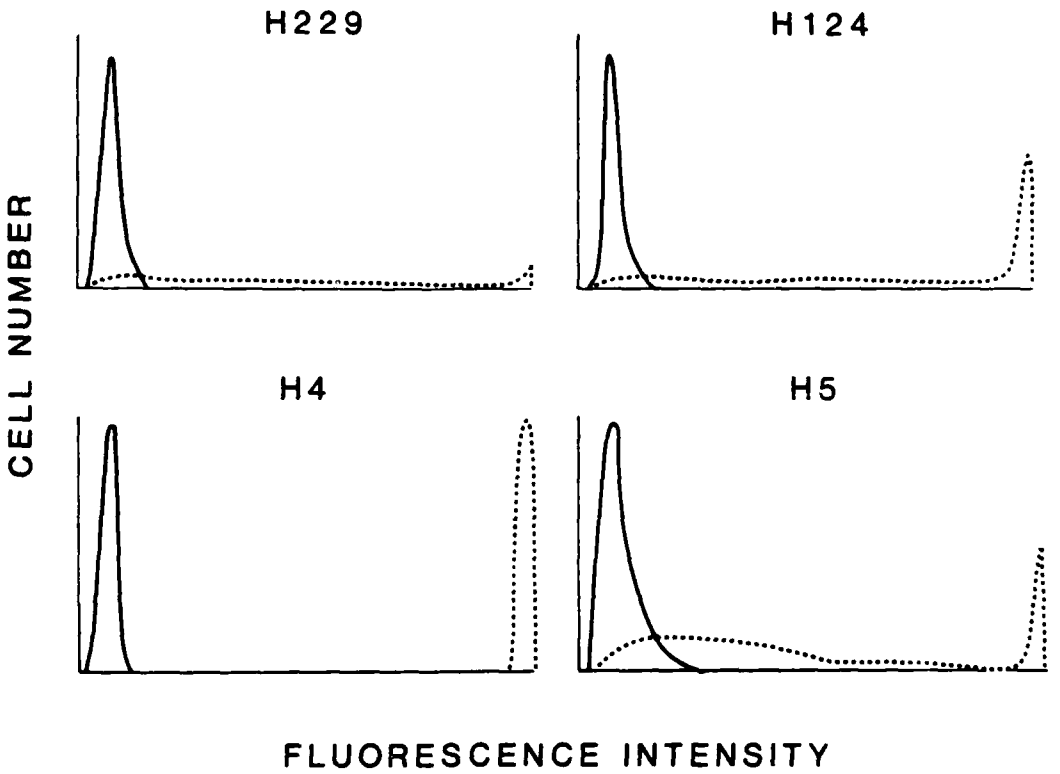

Figure C-2. Fluorocytometric analysis of platelet staining by mAb H229, H124, H4 and H5 ____ control mAb .... mAb under investigation.

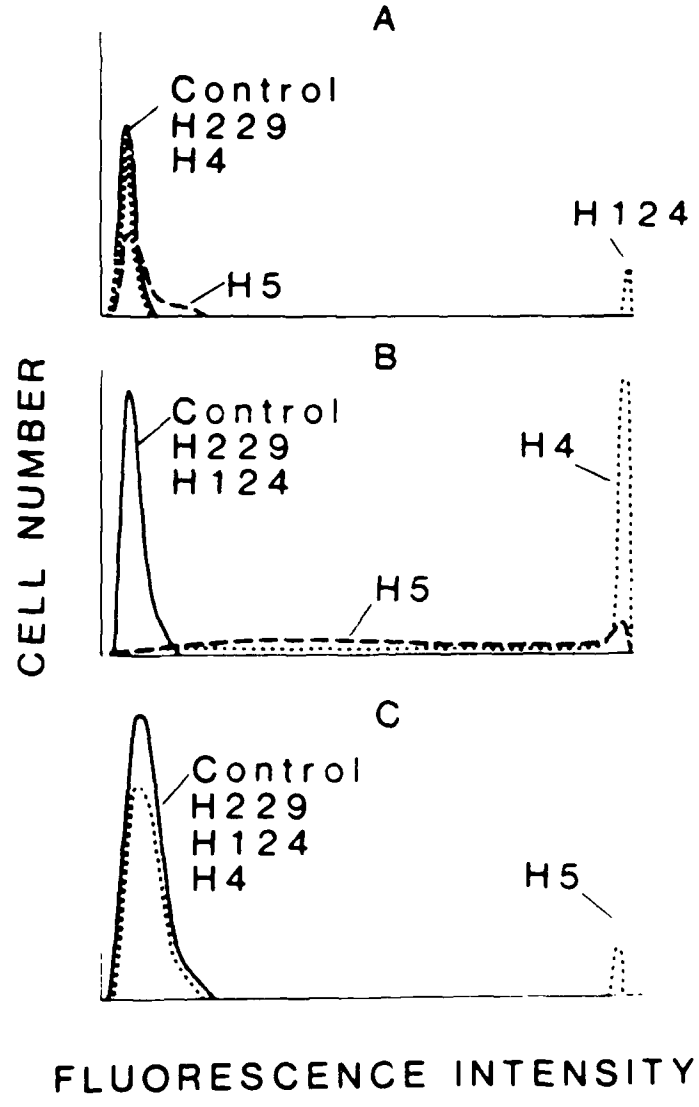

Figure C-3. Staining of lymphocytes (Panel A), monocytes (Panel B) and granulocytes (Panel C) as analysed by flow cytometry. A. ____ staining by control mAb, mAb H229 and H4; ----, mAb H5; and ...., mAb H124. B. ____ staining by control mAb, mAb229 and H124; ----, mAb H5; and ...., mAb H4. C. ____ control mAb, mAb H229, H124 and H4; and ...., mAb H5.

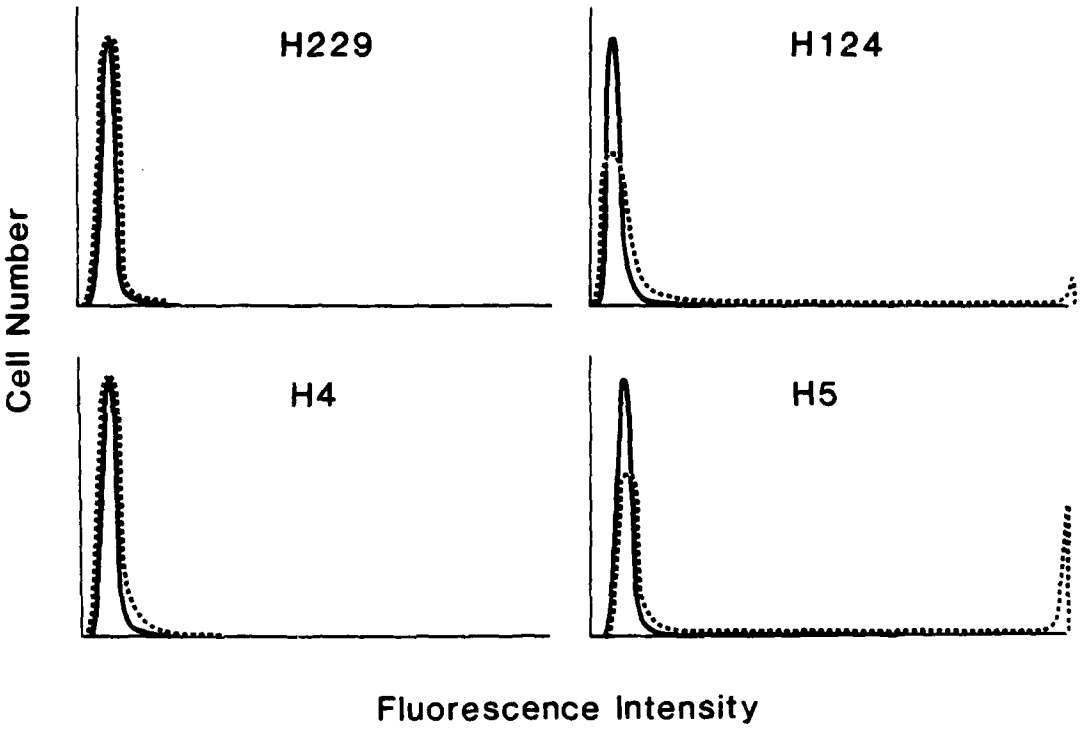

Figure C-4. Fluorocytometric analysis of staining patterns on small E^-sIg^-bone marrow cells by mAb H229, H124, H4 and H5. E^-sIg^-bone marrow cells were prepared by depletion of T cells with neuraminidase treated sheep red cells and by depletion of B cells by an immune rosette method using an anti-μ monoclonal antibody.

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